UNIVERSITY OF ALBERTA

PANCREATIC SECRETION IN PIGS

BY

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IN MEMORY OF REINHARD AND LILLIAN GABERT,

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ABSTRACT

A series of studies related to exocrine pancreatic secretion in pigs were carried out in order to determine the effect of diet composition and antinutritional factors on exocrine pancreatic secretions. In addition, studies were carried out to evaluate and compare two commonly used methods to collect pancreatic juice, namely the Pouch and Catheter Methods, and two different methods for assaying pancreatic enzymes. The inclusion of fababeans and peas in diets for young pigs did not affect pancreatic secretions in young pigs. Diet, 8-h sampling period and experimental period did not affect ($P > 0.05$) flows of total, protein-bound and free amino acids in pancreatic juice. In the third study, source of dietary fat (fish oil, rapeseed or coconut oil) did not affect ($P > 0.05$) specific or total lipase activity. A comparison of two commonly used methods to collect pancreatic juice indicated that there were differences ($P < 0.05$) in the volume of pancreatic juice, pH, secretion of protein, zinc and enzymes. The presence of active proteolytic enzymes in pancreatic juice from Pouch Method pigs was confirmed using electrophoresis. In an additional study, it was concluded that zinc excretion in pancreatic juice does not make a major contribution to zinc homeostasis in the pig. A comparison of two methods to assay pancreatic enzymes indicated that amylase, trypsin or chymotrypsin activity could be determined with either method.

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LIST OF ABBREVIATIONS NOT DEFINED IN THE TEXT

ABBREVIATION	DEFINITION
BW	Body Weight
°C	Degrees Celsius
CP	Crude protein (%N x 6.25)
cv	Cultivated variety
d	Day
DM	Dry Matter
g	Gravity
g	Gram
µg	microgram
GLM	General Linear Model
h	Hour
HPLC	High Performance (Pressure) Liquid Chromatography
IU	International Unit
L	Liter
µL	microliter
M	Molarity (moles L^{-1})
mg	Milligram
min	Minute
MJ	Megajoule
mL	Milliliter
mm	Millimeter
n	Number
N	Nitrogen
P	Probability

ABBREVIATION	DEFINITION
U	Unit
wt	Weight
vol	Volume

CHAPTER 1

GENERAL INTRODUCTION

A. Pancreatic Secretion in Pigs

The digestive tract of the pig enables it to digest and assimilate a wide variety of feedstuffs. The digestive system of the pig is highly developed and allows the pig to flourish in many areas of the world and become an integral part of our modern agricultural industry. The pancreas is an important part of the digestive system and it is the main source of digestive enzymes. The pancreas also secretes bicarbonate which neutralizes hydrochloric acid from the stomach thereby making the pH of duodenal contents slightly alkaline and favorable for digestion. The pancreas contains 90-95% exocrine tissue and only 2-3% endocrine tissue (Brannon 1990). The pancreas supplies enzymes needed for the digestion of lipids, carbohydrates and proteins (Rinderknecht 1993).

The secretions of the exocrine pancreas are required for digestion. These secretions include digestive enzymes in active or inactive form, pro-enzymes, polypeptide enzyme cofactors and inhibitors, mucins, bicarbonate, urea, sodium, potassium and chloride (Kidder and Manners 1978; Schulz 1987; Rinderknecht 1993). The regulation of secretion and activities of the various enzymes have been extensively studied (Solomon 1987; Corring et al. 1989; Chey 1993). The activities are highly dependent on diet composition, age, feeding regimen and time of sampling in relation to time of feeding (Corring and Saucier 1972; Hee

et al. 1988b). Adaptation of exocrine pancreatic secretion is likely necessary to optimize digestion. Exocrine pancreatic secretions have been shown to adapt to the level of protein, fat and starch in the diet (e.g., Corring 1980; Hee et al. 1988a; Ozimek et al. 1995). Pancreatic secretion can adapt by means of nonparallel secretions, i.e., where the proportions of pancreatic enzymes change (Davidson 1989). Pancreatic adaptation to a change in diet composition is usually complete within 5-7 d (Partridge et al. 1982; Corring et al. 1989).

Level and Source of Dietary Protein

In the pig, pancreatic secretion of proteolytic enzymes such as trypsinogen and chymotrypsinogen, which must be activated to trypsin and chymotrypsin, does respond to changes in the level of dietary protein. However, large differences in the protein content of the diet are needed to elicit this response (Corring et al. 1989). In the pig, specific activities of chymotrypsin and trypsin increased when the level of protein in the diet was increased from 0% (protein free) to 30%, whereas the concentration of protein in pancreatic juice did not change greatly (Corring and Saucier 1972). Hee et al. (1988a) fed protein-free diets and 14.5% CP diets; total trypsin activities increased from 115.3 to 243.4 U 24 h^{-1} x 10^{-3}, respectively, and total chymotrypsin activities increased from 73.8 to 166.2 U 24 h^{-1} x 10^{-3}, respectively.

The adaptation of pancreatic secretions to the level of dietary protein is due to the release of cholecystokinin from endocrine cells in the duodenum and upper jejunum which increases the secretion of proteolytic enzymes from the pancreas (Brannon 1990).

The quality of dietary protein can also affect pancreatic secretion. In the rat, increasing the intake of proteins with a more favorable amino acid balance can result in increased chymotrypsin secretion; this was not observed when gelatin or zein were fed unless the two most limiting amino acids (lysine and tryptophan) were supplemented (Brannon 1990). In studies with pigs fed either rapeseed concentrate or casein as the protein source, rapeseed concentrate decreased the volume of pancreatic juice and increased the concentration of protein in pancreatic juice (Valette et al. 1992). Rapeseed protein concentrate also increased the total activities of trypsin, chymotrypsin and carboxypeptidase B in pancreatic juice (Valette et al. 1992).

In conclusion, the pancreas can adapt to the level and quality of dietary protein. However, further studies with other protein sources are necessary to further characterize adaptation to protein quality.

Level and Source of Dietary Starch and Mono- and Disaccharides

The secretion of pancreatic amylase is very sensitive to changes in the level of starch in the diet. Studies by Ozimek et al. (1995) demonstrated that total amylase activity, in pancreatic juice from growing pigs, decreased when 15.0% corn starch was replaced by 15.0% canola oil. Similarly, Corring (1980) observed that when the level of starch in the diet was increased from 20 to 60%, there was a corresponding increase in specific activity of amylase in pancreatic juice. However, in a different study, as the level of starch in the diet was varied from 28.3 to 81.0%, the specific activity of amylase did not vary proportionately with the dietary level of starch (Corring and Saucier 1972). In the rat,

starch and glucose induce a similar increase in amylase secretion, however, sucrose and fructose increase amylase secretion to a lesser degree than starch (Brannon 1990).

The proposed mediator of amylase adaptation to the level of dietary starch and likely other carbohydrates is insulin. An increased plasma glucose level resulting from digestion and absorption of starch stimulates the release of insulin which regulates amylase synthesis and mRNA levels in acinar cells (Brannon 1990). Intracellular glucose alone or in conjunction with insulin, regulates amylase synthesis and mRNA levels (Brannon 1990).

Adaptation of pancreatic secretions to dietary carbohydrate occurs readily (Corring et al. 1989). There is, however, a scarcity of information on pancreatic adaptation to sources of carbohydrate other than starch in the pig. In addition, different sources of starch and different processing methods may elicit different responses.

Level and Source of Dietary Fat

Pancreatic secretions of lipase adapt to the level and source of dietary fat. Hee et al. (1988a) reported an increase in total lipase activity from 654 to 3950 U 24 h^{-1} x 10^{-3} when the level of dietary fat (tallow) was increased from 2 to 10%. In studies with pigs, when fat level was increased from 5 to 25%, lipase activity in pancreatic tissue increased by 83.0% and colipase activity increased by 37.5% (Mourot and Corring 1979).

In studies with rats, the effect of the degree of saturation and(or) chain length of dietary fatty acids on lipase activity is controversial. In some studies, unsaturated fat stimulated lipase secretion and long chain fatty acids increased lipase content in pancreatic tissue more than triglycerides with medium chain length (Brannon 1990).

Inclusion of polyunsaturated fatty acids increased lipase activity in pancreatic homogenate (Deschodt-Lanckman et al. 1971; Sabb et al. 1986; Ricketts and Brannon 1994).

With the exception of studies by Simoes-Nunes (1986), the pig has not been used as a model to study the effect of fatty acid composition on exocrine pancreatic secretions. Simoes-Nunes (1986) observed that pigs fed diets containing 21% sunflower oil had higher lipase activity in pancreatic homogenate than pigs fed diets with the same level of lard.

The quality of dietary fat has been shown to affect pancreatic secretions. Ozimek et al. (1995) observed that total activity of lipase in pancreatic juice increased (versus 15% canola) when 15% peroxidized canola oil was fed to growing pigs.

Secretin and ketones, fatty acid metabolites, are proposed mediators of pancreatic adaptation to dietary fat (Brannon 1990). Recent studies with rats suggest that the type of fat may act through a translational mechanism: moderate levels of saturated fat may reduce the efficiency of translation or decrease the synthesis of lipase through a pre-translational mechanism (Ricketts and Brannon 1994). Whether or not the same mechanism exists in the pig remains to be determined.

In pigs, there is a scarcity of information on the effect of fatty acid composition on pancreatic secretion of lipase as well as colipase, an essential cofactor for lipase activation. In addition, there is lack of information on the effect of the amount or source of dietary lipids on the activity of porcine carboxyl ester hydrolase, an enzyme that hydrolyses a variety of lipid substrates (Rinderknecht 1993). Therefore, studies were

carried out to investigate the effect of fatty acid composition on exocrine pancreatic secretion in growing pigs.

Dietary Fiber

Dietary fiber can affect pancreatic secretion by changing the physical properties of intestinal contents and absorptive processes. Ikegami et al. (1990) reported that volume of pancreatobiliary secretion and specific protease and amylase activities in rats increased when they were fed diets containing viscous polysaccharides including sodium alginate, locust bean gum, xanthan gum and guar gum. Weight of the pancreas and the small intestine were also increased and there was a positive relationship between specific amylase activity in pancreatobiliary secretion and viscosity in small intestinal contents. These observations suggest that the physical properties of fiber caused changes in pancreatic secretion. Feeding diets containing 5% pectin to rats (Forman and Schneeman 1980) also caused an increase in the volume of pancreatic juice likely through the effect of bulk. The activities of amylase, lipase, chymotrypsin and trypsin in the small intestine also increased (Forman and Schneeman 1980). The changes may have been due to increased volume, prevention of the degradation of digestive enzymes by pectin or an effect of pectin on increasing enzyme activity (Forman and Schneeman 1980). Similar results were presented by Schneeman et al. (1982).

Very few studies have been carried out in the pig. In studies by Zebrowska and Low (1987), when growing pigs were fed diets containing whole wheat or wheat bran, the volume of pancreatic juice was increased, compared to a control diet with a relatively low fiber content. Similar results were reported by Langlois et al. (1987); diets containing

40% wheat bran, fed to growing pigs with a catheter in the pancreatic duct, increased the volume of pancreatic juice, protein output and total chymotrypsin, trypsin, lipase and amylase activities. The increased volume of secretion was likely responsible for most of the increase in enzyme activities and was caused by an increase in secretin and vasoactive intestinal polypeptide levels (Langlois et al. 1987). In contrast, feeding diets containing pectin, Alphafloc or barley straw did not affect the volume of pancreatic juice secreted or total enzyme activities (Mosenthin and Sauer 1991; Mosenthin et al. 1994).

In conclusion, the level of fiber in the diet must be very high to affect pancreatic secretion in pigs. The effect of fiber source may also depend on its physical and chemical properties.

Tannins and Trypsin Inhibitors

Trypsin inhibitors and tannins are antinutritional factors (ANF) which may cause detrimental effects on the digestive system of animals. Marquardt et al. (1977) and Newton and Hill (1983) identified condensed tannins as the predominant ANF in fababeans, especially colored flowering (also referred to as dark) varieties. Tannins bind to proteins and enzymes making proteins resistant to digestion and inhibiting enzyme activity (Marquardt et al. 1977; Liener 1980). Tannins may also cause pancreatic hypertrophy and increase pancreatic secretion in animals but this has not been demonstrated in *in vivo* experiments (Huisman and Jansman 1991; Jansman 1993). In studies with 40 kg pigs, Jansman et al. (1994) observed that chymotrypsin and trypsin secretion were not affected when diets containing fababean hulls with a high content of condensed tannins were fed.

Trypsin inhibitors, present in soybeans, peas and other grain legumes, may increase pancreatic secretions in pigs (Huisman and Jansman 1991; Leterme et al. 1992). Li (1996) reported that raw Nutrisoy (defatted soybean flour) increased, compared to autoclaved Nutrisoy, the volume of pancreatic juice secreted in growing pigs and decreased the specific activities of amylase, trypsin and chymotrypsin. Hooks et al. (1965) fed diets containing 16 or 20% CP from raw soybean or solvent-extracted soybean meal to pigs and observed that the weight of the pancreas and the activities of proteolytic enzymes and lipase in pancreatic homogenate were not affected by diet. There was no effect of diet on cell structure or zymogen content of pancreatic tissue.

There is a scarcity of information on the effect of trypsin inhibitors on the volume of pancreatic juice, protein secretion and specific and total enzyme activities in pigs. There is also very limited information on the effect of tannins on exocrine pancreatic secretions in pigs. Therefore, studies were carried out to investigate the effect of feeding diets containing fababeans and peas to pigs fitted with a pancreatic pouch re-entrant cannula which enabled the total collection of pancreatic juice.

Methods to Collect Pancreatic Juice

To study exocrine pancreatic secretions and the effect of various dietary factors, hormones, agonists and inhibitors *in vivo*, surgical intervention is necessary to facilitate the collection of pancreatic juice. Two commonly used methods are employed to collect pancreatic juice in pigs. One method, referred to as the Catheter Method (CM), involves surgical placement of a catheter into the pancreatic duct and insertion of a small T-cannula into the duodenum to return collected pancreatic juice (Wass 1965; Corring et al. 1972;

Pierzynowski et al. 1988; Thaela et al. 1995). The second method, referred to as the Pouch Method (PM) also involves the permanent re-entrant diversion of pancreatic juice, however, pancreatic juice is collected from an isolated duodenal pouch in which the pancreatic duct enters (Hee et al. 1985; Li 1996). A variation of this method, which involves connecting the cannula in the duodenal pouch to a re-entrant duodenal cannula has also been used (e.g., Zebrowska et al. 1983; Zebrowska and Low 1987). Many studies have been performed, using either method, to investigate the effects of different levels and sources of dietary protein, starch, fat and fiber on pancreatic secretions (Partridge et al. 1982; Hee et al. 1988a; Valette et al. 1992; Mosenthin et al. 1994; Li 1996).

In the construction of the isolated duodenal pouch, most of the extrinsic nerves leading from the stomach to the pancreas via the duodenum must be severed; regulatory mechanisms may be affected and pancreatic secretion altered (Thomas 1959; Pierzynowski et al. 1988). The presence of a catheter in the pancreatic duct may also affect secretion. When a catheter is placed in the pancreatic duct, the duct is ligated just before entry into the duodenum and the duodenal papilla bypassed. The duodenal papilla functions as a one-way valve to prevent the backflow of pancreatic juice and duodenal digesta and likely provides some, as yet an undetermined amount of resistance, to the flow of pancreatic juice through the ductal system. Removal of the function of the papilla may increase the secretion of pancreatic juice. Comparing results obtained with the two methods in independent experiments is difficult as they may be confounded by different experimental conditions (Partridge et al. 1982). An implicit assumption is that pancreatic secretions respond to changes in the amount and type of dietary nutrients in the same way regardless which

surgical technique is used. It is also assumed that the regulatory mechanisms of pancreatic secretions are maintained and not affected by collection method.

Each method used for preparing pigs for total collection of pancreatic juice has its strengths and weaknesses. The Pouch Method can be used in long term experiments and survival rate and complications may be less than when the Catheter Method is used (Hee et al. 1985). However, recovery time from surgery is longer and severing the intestine to create a duodenal pouch may affect the regulation of pancreatic secretions (Pierzynowski et al. 1995). The Catheter Method can be used in short term studies, however, there is a greater chance that complications will develop (Partridge et al. 1982; Hee et al. 1985).

A comparison of the two most commonly used methods to collect pancreatic juice from pigs is required. Therefore, studies were carried out to compare the volume of pancreatic juice, pH of pancreatic juice, protein, bicarbonate and zinc secretions and specific and total enzyme activities in pancreatic juice collected with the Pouch or Catheter Method.

Storage, Freezing and Thawing of Pancreatic Juice

Refrigerated or frozen storage of samples is often required due to technical limitations before determining enzyme activities in pancreatic juice, especially if a large number of samples are to be analyzed. Changes in enzyme activities during storage could lead to misinterpretation of results. Repeated freezing and thawing of samples may be necessary if the researcher decides to do additional analyses. Gorrill and Thomas (1967) reported that repeated freezing and thawing of nonactivated and undiluted bovine pancreatic juice did not decrease trypsin and chymotrypsin activities. Makkink et al. (1990) observed

that storage of pancreatic juice for 3 wk at -20 or -80°C did not affect the activities of trypsin, chymotrypsin or lipase. However, enzyme activities declined when pancreatic juice was stored at 4°C.

Most studies involving storage have been carried out with samples of human duodenal aspirate which contains active proteolytic enzymes. However, a description of the exact procedures of thawing duodenal aspirate prior to assay have not been reported. This is an important consideration that needs to be investigated further and if at all possible, thawing procedures should be standardized. Lipase activity did not decrease during storage at -15°C in the studies of Borgström and Hildebrand (1975), however, Kelly et al. (1991) reported that lipase activity steadily declined during storage at -20°C while chymotrypsin and to a lesser extent trypsin activities were quite stable for 7 wk. Amylase, lipase and trypsin activities in duodenal aspirate were quite stable at -20°C for 4 wk (Legg and Spencer 1975). However, when duodenal aspirate was stored at 4°C or room temperature, large decreases in lipase activity were observed but there were much smaller decreases in amylase and trypsin activities (Legg and Spencer 1975).

Adequate storage of samples is required to prevent changes in chemical composition and enzyme activities in pancreatic juice. However, there is a scarcity of information on the effect of freezing, storage and thawing on the activities of enzymes. Therefore, studies were carried out to investigate the effect of freezing and thawing on enzyme activities.

B. Objectives of Thesis

The objectives and related hypothesis of studies in this thesis were as follows:

1. Objective: To investigate the effect of including whole fababeans (37.7% in the diet), which supplied 50% of dietary CP and had a relatively high content of condensed tannins, on exocrine pancreatic secretions of N, protein and enzymes in young pigs. The control diet contained soybean meal as the sole source of dietary protein. A second objective was to determine the effect of including dry peas as the sole source of dietary protein, with a relatively high content of trypsin inhibitors, on exocrine pancreatic secretions of N, protein and enzymes in young pigs. The control diet contained a variety of peas with a relatively low content of trypsin inhibitors.

Hypothesis: Inclusion of whole fababeans, with a relatively high content of condensed tannins, and dry peas, with a relatively high content of trypsin inhibitors, in diets for young pigs would increase the volume of pancreatic juice secreted and(or) increase the secretion of proteolytic enzymes such as trypsin and(or) chymotrypsin.

2. Objective: To determine whether or not the concentration and flows of total, protein-bound and free amino acids (AA) in pancreatic juice were affected by diet, sampling time and age (BW). An additional objective was to determine the relative proportions of free and protein-bound AA in relation to total AA and to examine the relationship of AA flows to secretion of enzymes in pancreatic juice.

Hypothesis: Pancreatic juice would contain a high level of protein-bound AA and the concentration of protein-bound AA would be directly related to the secretion of pancreatic enzymes.

3. Objective: To investigate the effect of dietary fat source on the volume of pancreatic juice secreted and the secretion of protein and enzymes responsible for fat digestion: lipase, colipase and carboxyl ester hydrolase, in pancreatic juice collected from growing pigs. The dietary fat sources were fish oil, rapeseed oil (extracted from low erucic acid and low glucosinolate rapeseed) and coconut oil. Diets containing 15% of these fat sources were fed to pigs prepared for collection of pancreatic juice with the Pouch or Catheter Method.

Hypothesis: The diets containing fish oil and rapeseed oil, which contain more unsaturated fatty acids than coconut oil, would stimulate lipase and colipase secretion in pancreatic juice. In addition, the effect of fat source would be similar regardless of which surgical method was used to prepare the pigs for collection of pancreatic juice.

4. Objective: To evaluate two methods which are used for total collection of pancreatic juice in pigs. These methods are referred to as the Pouch and Catheter Methods, respectively. Pigs with a similar genetic background and age (BW) were kept under the same experimental conditions and volume of pancreatic juice, pancreatic secretions of bicarbonate, protein and enzymes were compared in the two groups of pigs.

Hypothesis: The volume of pancreatic juice, secretion of bicarbonate, protein and enzymes would be similar regardless of which method was used to collect pancreatic juice. The

diurnal pattern of secretion of pancreatic juice and the pH of pancreatic juice was expected to be similar in pigs prepared with either method.

5. Objective: To compare the daily secretion of zinc in pancreatic juice to daily zinc intake and to compare the diurnal pattern of the secretion of protein, zinc and carboxypeptidase A and B in pigs prepared with the Pouch or Catheter Method. A third objective was to determine the relationship between zinc secretion and carboxypeptidase A and B activities in pancreatic juice.

Hypothesis: Daily zinc secretion in pancreatic juice would be relatively high compared to daily zinc intake. The diurnal pattern of the secretion of protein, zinc and carboxypeptidase A and B would be similar regardless of the method used to collect pancreatic juice from pigs. Furthermore, there would be a high correlation between zinc secretion in pancreatic juice and carboxypeptidase A and B activities.

6. Objective: To compare two different methods for measuring the activities of amylase, lipase, trypsin and chymotrypsin activities in pancreatic juice. A second objective was to investigate the effect of thawing frozen samples of pancreatic juice on enzyme activities in order to determine the stability of pancreatic enzymes.

Hypothesis: There would be a high correlation between two different methods used to measure the activities of amylase, lipase, trypsin and chymotrypsin in pancreatic juice. In addition, thawing samples of pancreatic juice would not affect enzyme activities, i.e., enzymes in pancreatic juice are stable and are not affected by handling.

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CHAPTER 2

EXOCRINE PANCREATIC SECRETION OF NITROGEN, PROTEIN AND ENZYMES IN YOUNG PIGS FED DIETS CONTAINING FABABEANS AND PEAS[1]

A. Introduction

Increasing production of fababeans (*Vicia faba*) and white-flowering spring peas (*Pisum sativum* ssp. *sativum*) has created interest among the feed industry and swine producers to use these grain legumes in diets for pigs. However, there is a scarcity of information on the suitability of these legumes for use in diets for young pigs. Marquardt et al. (1977) and Newton and Hill (1983) identified condensed tannins as the predominant antinutritional factors (ANF) in fababeans, especially in colored flowering (also referred to as dark) varieties. Condensed tannins are found primarily in the hull (testa) fraction of fababeans (Jansman 1993). Tannins bind to proteins and enzymes making proteins resistant to digestion and inhibiting enzyme activity (Marquardt et al. 1977; Liener 1980). Studies by Grala et al. (1993) with 20-kg pigs demonstrated that condensed tannins in whole fababeans can decrease apparent ileal amino acid digestibilities and N retention. Other ANF, such as hemagglutinins (lectins) and trypsin inhibitors (Marquardt et al. 1975) and vicine and

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convicine (Frölich and Marquardt 1983), are also present in fababeans. In contrast, dry peas, from white-flowering varieties, usually contain very low levels of tannins (Savage and Deo 1989). However, some varieties may contain relatively high levels of trypsin inhibitors (Leterme et al. 1992). Antinutritional factors, such as tannins and trypsin inhibitors may also cause pancreatic hypertrophy and increase pancreatic secretion (Huisman and Jansman 1991; Jansman 1993) although this remains to be demonstrated in studies with pigs. In studies with 40-kg pigs fitted with a pancreatic pouch re-entrant cannula, Jansman et al. (1994) observed that chymotrypsin and trypsin secretion were not affected when diets containing fababean hulls with a high content of condensed tannins were fed. The Pouch Method, which involves the permanent re-entrant diversion of pancreatic juice, was initially developed by Hee et al. (1985) and was later used in several studies with growing pigs (e.g., Mosenthin and Sauer 1991; Pöhland et al. 1993; Jansman et al. 1994). The Pouch Method was adapted for use with young pigs in the present investigation. This is the first time the Pouch Method has been used to collect pancreatic juice in young pigs.

The first objective of this study was to investigate the effect of including whole fababeans (37.7% of the diet and supplied 50% of dietary CP), which had a relatively high content of condensed tannins, on exocrine pancreatic secretions of N, protein and enzymes in young pigs. The control diet contained soybean meal as the sole source of dietary protein. The second objective of this study was to determine the effect of including peas as the sole source of dietary protein, with a relatively high content of trypsin inhibitors, on exocrine pancreatic secretions of N, protein and enzymes in young pigs. The control diet contained a variety of peas with a relatively low content of trypsin inhibitors.

B. Materials and Methods

Cannula Preparation

The cannulas (Fig. 2-1) were designed and prepared in a different manner than described by Hee et al. (1985). Cannulas were prepared from Plastisol (Techniplast Plastisol CA 1098 Polyvinylchloride Clear, F. H. & Sons Mfg. Ltd., Rexdale, ON) using a carbon steel mould and the following conditions. Approximately 0.1 g of silicone grease (Dow Corning High Vacuum Grease Silicone Lubricant, Dow Corning, Midland, MI) was evenly applied to the mould to prevent adherence of Plastisol. The mould was preheated at 300°C for 15 min in an ashing oven. The mould was immersed into Plastisol for 0.5-1 min and returned to the furnace for 2-3 min. This procedure was repeated several times until the thickness of the cannula was 4 mm. The retaining rings (thickness 5 mm) were prepared by pouring Plastisol into an aluminum dish and heating at 300°C for 3 to 4 min. The cannulas were connected with a plastic one-way valve (catalog no. 15-339-2, Fisher Scientific, Ottawa, ON).

Animals and Diets

The pigs (Camborough x Canabrid) were obtained from the University of Alberta swine herd. The pigs were housed individually in metabolic crates in a temperature-controlled room (24-25°C). Water was freely available from a low-pressure drinking nipple.

Experiment 1

Eight barrows were used in Experiment 1 and the pigs were weaned at 28 d of age. The weaning weight of the pigs was 7.6 kg and surgery was conducted 7 d after weaning.

Seven days prior to surgery the pigs had free acces to a wheat-soybean meal diet which contained 20% CP. Prior to surgery, the average BW of the pigs was 8.1 kg.

The pigs were fitted with a pancreatic pouch re-entrant cannula (Fig. 2-1) according to procedures adapted from Hee et al. (1985) with some modifications. The duodenal T-cannula was inserted approximately 20 cm posterior to the site of anastomosis. This was done to avoid adhesions that would be caused if the cannula was placed in close proximity to the anastomosis site and to increase the space available in which to carry out the surgical procedures. The pouch cannula was exteriorized between the third and fourth last rib; the duodenal T-cannula was exteriorized behind the last two ribs. Following surgery the pigs had free access to water but were starved that same day. The next day, the pigs were fed 150 g, prior to the start of Experiment 1, in three meals of equal amount at 0800, 1600 and 2400 h. The pigs were allowed a 7-d recuperation period and the daily intake was increased gradually until the pigs consumed approximately 340 g d^{-1}.

Two corn starch-based diets were formulated to contain 20% CP. The formulation of the diets is presented in Table 2-1. In one of the diets, referred to as the SBM diet, soybean meal was the sole protein source. In the other diet, referred to as the SF diet, soybean meal (22.2%) and dry whole fababeans (cv Fibro; colored-flowering; 37.7%) each supplied 50% of the dietary CP. This cultivated spring variety is known to have a relatively high content of condensed tannins (Mosenthin et al. 1993). The soybean meal and fababeans were ground through a 3-mm mesh screen prior to incorporation into the diets. Dextrose (10.0%) was included to improve palatability. Canola oil was included to reduce dustiness. Vitamins and minerals were supplemented to meet or exceed National Research

Council standards (NRC 1988). Four pigs were randomly assigned to each experimental diet. Each experimental period consisted of 8 d: 6 d adaptation followed by a 2-d collection of pancreatic juice. The pigs were fed 340 g d^{-1} (4% of the average BW determined 12 h prior to the start of period 1) in three meals of equal amounts at 0800, 1600 and 2400 h. The average BW of the pigs at the start of period 1 and at the conclusion of the experiment were 8.5 and 9.9 kg, respectively.

Experiment 2

Five barrows were used in Experiment 2 and the pigs were weaned at 28 d of age. The initial BW of the pigs was 15.8 kg and they were 42 d of age when obtained from the herd. Surgery was conducted 7 d after the pigs were removed from the herd. Seven days prior to surgery, the pigs were given free access to a 20% CP wheat-soybean meal diet. The average BW of the pigs prior to surgery was 17.4 kg.

The pigs were fitted with a pancreatic pouch re-entrant cannula according to procedures described under Experiment 1. Following surgery, the pigs had free access to water but were starved that same day. The next day, the pigs were fed 195 g, prior to the beginning of Experiment 2, in three meals of equal amount at 0800, 1600 and 2400 h. Daily feed intake was gradually increased until the pigs consumed approximately 900 g d^{-1}. The pigs were allowed a 7-d recuperation period.

Two diets were formulated to contain 15.0% CP with dry whole peas as the sole protein source. The formulation of the diets is presented in Table 2-1. White-flowering spring peas were used and the pea cultivars selected were Ascona (yellow seeded) and Radley (green seeded) which have relatively low and high trypsin inhibitor activities with

respect to other white-flowering spring cultivars (Leterme et al. 1992). The diets containing peas are referred to as the AP and RP diets, respectively. The peas were ground through a 3-mm mesh screen prior to incorporation into the diets. Corn starch, dextrose and canola oil were included in the diets as in Experiment 1. Vitamins and minerals were supplemented to meet or exceed National Research Council standards (NRC 1988). A two-period change-over design was used; in the first experimental period, two pigs were randomly assigned to the AP diet and three to the RP diet. In the second experimental period the pigs were changed-over and received the other experimental diet. Each experimental period consisted of 8 d: 6-d adaptation followed by a 2-d collection of pancreatic juice. The pigs were fed 900 g d^{-1} (5% of the average BW prior to the start of period 1) in periods 1 and 2. The pigs were fed three meals of equal amounts at 0800, 1600 and 2400 h. The average BW of the pigs at the start of period 1 and at the conclusion of the experiment were 18.1 and 22.6 kg, respectively.

Collection of Pancreatic Juice

The one-way valve connecting the pouch and duodenal T-cannula was flushed with water every other day to ensure it was not blocked and there was no backflow. Medical tape (1.5 cm wide) was used to secure the retaining rings and to prevent the cannulas from disconnecting. Udderfax™ (a zinc oxide and lanolin-based cream, Coopers Agrofarm, Ajax, ON) was liberally applied around the cannulas after washing to minimize skin irritation.

Pancreatic juice was collected on d 7 and 8 for a total of 24 h from 0800 to 1600 h on d 7, 2400 h on d 7 to 0800 h on d 8 and 1600 to 2400 h on d 8, respectively. At the start of each 8-h sampling period, the cannulas were disconnected and a pipe clamp was placed

approximately 1-2 mm from the distal end of the pouch cannula. Soft plastic tubing (internal diameter 3 cm; length 15 cm), heat-sealed at one end, was fastened to the barrel of the cannula with a twist tie. When the tubing was approximately half full, pancreatic juice was emptied into a 500 mL-Erlenmeyer flask kept in a circulating water bath (38-39°C). After each hour of collection, pancreatic juice was transferred to a graduated cylinder. The volume was measured and 20 and 10% of the total volume collected was transferred to a 15-mL plastic vial in Experiments 1 and 2, respectively. The volume sampled was replaced with saline (154 mM sodium chloride, 38-39°C) and the remaining pancreatic juice was slowly infused into the duodenal T-cannula using a 140-mL syringe. A smaller proportion was sampled in Experiment 2 due to the larger volume of secretion. Pancreatic juice was immediately frozen at -27°C. The next day the samples were transported to the laboratory and stored at -50°C.

Chemical Analyses

Diets and ingredients were ground through a 0.8-mm mesh screen and mixed before analyses. Analyses for DM, CP, ether extract and ash were carried out according to Association of Official Analytical Chemists (1990) methods. The energy contents of the diets and ingredients were measured with a Parr Adiabatic Bomb Calorimeter (Parr Instrument, Moline, IL).

For amino acid analyses, approximately 100 mg of sample was weighed into 13 x 100 mm screw-capped test tubes, mixed with 6 mL of 6 M hydrochloric acid, flushed with N_2, capped and hydrolyzed at 110°C for 24 h. Analyses for amino acids were carried out by a fluorometric method involving pre-column derivatization with *o*-phthaldialdehyde

according to Jones and Gilligan (1983) using the HPLC system described by Dugan et al. (1989). Peaks were recorded and integrated with the EZchrom™ Chromatography Data System (version 2.12, Shimadzu Scientific Instruments, Columbia, MD). Methionine, cysteine, tryptophan and proline were not determined.

Tannins were measured as catechin equivalents using the vanillin-sulfuric acid method of Kuhla and Ebmeier (1981). Trypsin inhibitor activity (TIA) was assayed as described by van Oort et al. (1989).

The hourly pancreatic juice samples were thawed at 5°C and proportionally pooled within each 8-h sampling period to provide a pooled sample volume of 12 mL. All manipulations were performed in a 5°C walk-in fridge. The pooled sample was stirred; seven minivials were labelled and 1 mL of the pooled sample was pipetted into each vial. The remainder of the samples and all minivials were frozen at -50°C. Prior to performing each assay, one set of subsamples (1 mL) was thawed at 5°C.

Total N in pancreatic juice was measured with a Leco® FP-428 N Determinator (Leco®, St. Joseph, MI). The concentration of protein in pancreatic juice was determined according to the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical, St. Louis, MO, code: P 0914, 1 mg mL^{-1} in 0.15 M sodium chloride) as a standard.

Amylase activity was determined according to procedures described by Rick and Stegbauer (1974) and Worthington Biochemical Corporation (1988); lipase activity was determined according to Schmidt et al. (1974).

Activation using porcine enterokinase (enteropeptidase, Sigma, salt free lyophilized powder, code: E 0632) of chymotrypsinogen and trypsinogen to chymotrypsin and trypsin, respectively, was carried out according to Gorrill and Thomas (1967) and Glazer and Steer (1977) with some modifications. Firstly, 0.2 mL of undiluted pancreatic juice were added to 1.8 mL buffer (pH 8.1) containing 100 μg mL^{-1} bovine serum albumin (Sigma, code: A 3059), 50 mM calcium chloride and 50 mM tris(hydroxymethyl)aminomethane (Sigma 7-9®, code: T 1378). Enterokinase was dissolved in distilled and deionized water (10 mg mL^{-1}) and centrifuged at 15,600 x *g* and 5°C for 15 min to remove cell debris. Activation was initiated by adding 0.2 mL of the diluted pancreatic juice to 0.2 mL of enterokinase supernatant followed by incubation at 5°C for 3 and 120 h for chymotrypsin and trypsin, respectively. Chymotrypsin and trypsin activities were measured according to Rick (1974a,b).

The enzyme activity in pancreatic juice was expressed as units (U) x 10^{-3} per liter (specific activity) and per 8 or 24 h (total activities). One U of enzyme activity was defined as the hydrolysis of 1 μmol of substrate in 1 min. Total enzyme activities were calculated as follows: specific activity x volume of pancreatic juice secreted per 8 or 24 h.

Statistical Analyses

A one-way analyses of variance was carried out in Experiment 1 and diet was the source of variation. In Experiment 2, analyses of variance was conducted according to a two-period changeover design (Gill and Magee 1976) with pig as block and period and diet as main effects using the GLM Procedure of the SAS Institute, Inc. (1988). Volume of pancreatic juice, N and protein concentration and secretion as well as specific and total

enzyme activities during the three 8-h sampling periods were analyzed as repeated measures (Gill and Hafs 1971) using the GLM procedure of the SAS Institute, Inc. (1988). However, test for sphericity ($P > 0.05$) for some parameters indicated that F values from split-plot analyses were valid (SAS Institute, Inc. 1991). The error term period x diet x pig was used to test the effects of period, diet and pig in the whole plot. Time (8-h sampling period) and diet x time were included in the split-plot. Where appropriate, means were compared using the Student-Newman-Keuls' multiple range test (Steel and Torrie 1980).

C. Results

The pigs remained healthy and consumed their meal allowances throughout each experiment. Postmortem examinations, carried out at the conclusion of each experiment, revealed no intestinal adhesions.

The chemical composition, tannin contents and TIA values for soybean meal, fababeans, and the diets fed in Experiment 1 are presented in Table 2-2. The proximate and amino acid composition of the diets were similar to the expected values calculated from the composition of the ingredients and their level in the diets thus indicating accurate mixing and analyses.

The effect of diet on the volume of pancreatic juice secreted and concentrations and flows of N and protein and specific and total enzymes in Experiment 1 are shown in Table 2-3. There was no effect ($P > 0.05$) of diet on the volume of pancreatic juice secreted, N and protein concentrations and specific or total activities of amylase, lipase and chymotrypsin. There was no effect ($P > 0.05$) of 8-h sampling period on the volume of pancreatic juice

secreted, N and protein concentrations and specific or total enzyme activities. Therefore, total 24-h enzyme activities are presented for the diets. Specific trypsin activity was higher ($P < 0.05$) in pancreatic juice from pigs fed the SF than the SBM diet (53.7 versus 41.2 U L^{-1} x 10^{-3}). However, total trypsin activity did not differ ($P > 0.05$) in pancreatic juice of pigs fed the SBM or SF diets.

The chemical composition, tannin contents and TIA values for peas and the diets fed in Experiment 2 are presented in Table 2-2. The proximate and amino acid composition of the diets were similar to the expected values calculated from the composition of the ingredients and their level in the diets.

In Experiment 2, there was no effect ($P > 0.05$) of diet or 8-h sampling period on the volume of pancreatic juice secreted, N and protein concentrations and specific or total enzyme activities (Table 2-3). Therefore total 24-h enzyme activities are presented.

The effect of experimental period on the volume of pancreatic juice secreted and the concentrations and flows of N and protein and specific and total enzyme activities in pancreatic juice from pigs in Experiment 2 is shown in Table 2-4. There was no effect ($P > 0.05$) of experimental period on the volume of pancreatic juice secreted. However, the concentration of N and the concentration and 8 h-flow of protein were greater ($P < 0.05$) in period 2 than in period 1. These differences were not apparent when the secretion rates were expressed over 24 h due to the lower number of observations and subsequently higher variation. The specific activity of amylase was higher in period 2 ($P < 0.05$) than in period 1. The total (8 h) activities of amylase, lipase, chymotrypsin and trypsin where higher ($P < 0.05$) in period 2 than in period 1.

D. Discussion

The content of condensed tannins in the fababeans used in this study (Table 2-2) is similar to that reported by Mosenthin et al. (1993) and within the lower range of values reported by Jansman (1993). As expected, the tannin content of soybean meal is relatively low. Subsequently the tannin content of the SBM diet is very low. The TIA values for soybean meal and fababeans are in agreement with values reported by Leterme et al. (1990) and Huisman and Jansman (1991).

The lack of effect of the 8-h sampling periods is in agreement with the results of Hee et al. (1988); there were no differences ($P > 0.05$) in volume, protein or enzyme secretions between two 12-h periods (0800 to 2000 h and 2000 to 0800 h).

The inclusion of 37.7% whole fababeans in the SF diet did not affect ($P > 0.05$) the volume of pancreatic juice secreted by pigs in Experiment 1 (Table 2-3). In studies by Jansman et al. (1994) the volume of pancreatic juice secreted was also not affected ($P > 0.05$) when growing pigs were fed diets containing fababean hulls with a high level of tannins. The increase in specific trypsin activity in pancreatic juice from pigs fed the SF diet is of minor nutritional significance as total enzyme activity reflects the quantity of enzymes that can participate in digestion. Total trypsin activity was not affected ($P > 0.05$) by diet (Table 2-3). These results are in agreement with those reported by Jansman et al. (1994). When growing pigs were fed diets containing fababean hulls with a high tannin content there was no effect ($P > 0.05$) on the total activities of trypsin or chymotrypsin. This may indicate that very high levels of tannins have to be included in the diet to elicit a response or that the digestive system responds to these ANF by simply increasing the volume of

pancreatic juice. In addition, as was discussed by Mosenthin and Sauer (1991), these results illustrate that it is essential to consider total rather than specific enzyme activities when studies are carried out to determine the effect of diet composition on pancreatic enzymes.

The TIA values for the dry peas used in Experiment 2 (Table 2-2) are within the range of values reported by Leterme et al. (1992) for spring cultivars; as expected, the value for cv Radley was higher than for cv Ascona. In addition, the tannin contents of peas were quite low; white-flowering varieties of peas have very low tannin contents (Huisman and Jansman 1991). Consequently, the tannin contents of the pea diets were very low. The lack of an effect of trypsin inhibitors in peas was likely due to their relatively low level in the pea diets (Table 2-2).

The volume of pancreatic juice secreted by pigs in Experiment 2 was approximately three times higher than in Experiment 1 (Table 2-3). These studies confirm the results of Weström et al. (1988); as BW (age) increases, secretion rate increases. The volume of pancreatic juice secreted by pigs in Experiment 2 approached the value of 2984 mL 24 h^{-1} which was the volume secreted by 40-kg pigs fed corn starch-based soybean meal diets in studies by Pöhland et al. (1993).

The concentration of N in pancreatic juice from pigs in Experiment 1 was approximately three times higher than in Experiment 2 (Table 2-3). However, total daily N flows were similar due to the higher flow of pancreatic juice in Experiment 2. Total daily N secretions in both experiments were lower than the 2.71 g 24 h^{-1} reported for 40-kg pigs by Pöhland et al. (1993) but similar to 1.3 g 24 h^{-1} determined in 48-kg pigs by Partridge et al. (1982).

Protein concentration in pancreatic juice was higher in Experiment 1 than in Experiment 2 (Table 2-3). However, the secretions of protein over 24 h were similar in both experiments.

Specific and total amylase activities in Experiment 1 were approximately eight and three times greater than those in Experiment 2 (Table 2-3), respectively. This was most likely due to the higher starch (amylose) content of the diets fed in Experiment 1 (35.4 and 20.1% for the SBM and SF diets, respectively; Table 2-1) than in Experiment 2 (12.6 and 9.8%, excluding pea starch, for the AP and RP diets, respectively; Table 2-1). These results are in agreement with previous studies which have shown that the secretion of pancreatic amylase is very sensitive to changes in the dietary content of starch. Studies by Ozimek et al. (1995) demonstrated that total pancreatic amylase activity decreased when 15.0% corn starch was replaced by 15.0% canola oil. In studies with rats (Snook and Meyer 1964), differences in dietary starch intake were also reflected by corresponding changes in amylase secretion in pancreatic juice. Lower specific amylase activities were reported in previous studies in which diets with higher inclusion levels of starch were fed to growing pigs. Mosenthin and Sauer (1991) and Pöhland et al. (1993) reported activities of 482 and 89 U L^{-1} x 10^{-3}, respectively, when diets containing 49.9 and 48.3% corn starch were fed. The different specific amylase activities were likely due to several factors. As was pointed out by Imbeah et al. (1988) it is rather difficult to compare results from different studies as these are confounded by differences in feed intake, feeding regimen, diet composition, BW and different techniques that are used to collect pancreatic juice.

The specific lipase activities were higher in Experiment 1 than in Experiment 2 but total lipase activities were similar (Table 2-3). The ether extract contents of the diets fed in both experiments were similar (Table 2-2). Higher specific activities were reported in previous studies with growing pigs. Hee et al. (1988) and Mosenthin and Sauer (1991) reported activities of 40 and 58 U L^{-1} x 10^{-3} when diets containing 2.0% tallow and 4.0% canola oil were fed, respectively. The differences in lipase activities between this study and the aforementioned studies were probably due, in part, to differences in BW between the pigs.

The specific chymotrypsin and trypsin activities were higher in Experiment 1 than in Experiment 2 but were similar to the specific activity values reported by Mosenthin and Sauer (1991).

It is concluded from the results of Experiment 1 that inclusion of 37.7% of a colored-flowering spring variety of fababeans, which had a relatively high content of condensed tannins, in a diet for young pigs does not affect exocrine pancreatic secretions of protein and total enzyme activities. From the results of Experiment 2, it is concluded that the inclusion of 70.5% of a white-flowering spring variety of peas, with a relatively high content of trypsin inhibitors, in a diet for young pigs does not affect exocrine pancreatic secretions of protein and enzymes.

E. Implications

The results of this study indicate that condensed tannins in a colored-flowering variety of fababeans, when whole beans are included in the diet at a level of 37.7%, do not

have any detrimental effects on exocrine pancreatic secretions in young pigs. Furthermore, when a white-flowering spring cultivar of peas, with a relatively high content of trypsin inhibitors, is included in a diet for young pigs (70.5% dry peas), trypsin inhibitors do not have any negative effects on exocrine pancreatic secretions in young pigs.

Table 2-1. Formulation (%) of the experimental diets

	Experiment 1		Experiment 2	
Ingredients	SBM[z]	SF	AP	RP
Soybean meal	44.4	22.2		
Fababeans		37.7		
Peas (cv Ascona)			67.6	
Peas (cv Radley)				70.5
Corn starch	35.4	20.1	12.6	9.8
Dextrose	10.0	10.0	10.0	10.0
Canola oil	3.0	3.0	3.0	3.0
Solkafloc[y]	3.0	3.0	3.0	3.0
Dicalcium monophosphate	1.7	1.4	1.1	1.0
Calcium carbonate	1.1	1.2	1.0	1.0
Vitamin and mineral premix[x]	1.0	1.0	1.0	1.0
Trace-mineralized salt[w]	0.4	0.4	0.5	0.5
DL-Methionine (98%)			0.2	0.2

[z]SBM, soybean meal as protein source; SF, 50% crude protein from soybean meal and 50% crude protein from fababeans (cv Fibro); AP, cv Ascona peas as protein source, RP, cv Radley peas as protein source.

[y]Supplied by Brown Co., Berlin, NH.

[x]The vitamin and mineral premix provides the following per kilogram of diet: vitamin A, 5000 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; choline, 600 mg; niacin, 45 mg; d-pantothenic acid, 25 mg; riboflavin, 12 mg; d-biotin, 0.2 mg; folic acid, 0.2 mg; vitamin B_{12}, 0.03 mg; Fe, 150 mg; Cu, 125 mg; Zn, 120 mg; Mn, 20 mg; Se, 0.3 mg; I, 0.2 mg.

[w]Supplied by Windsor Salt Co., Toronto, ON. Composition (%): NaCl, 96.5; ZnO, 0.40; $FeCO_3$, 0.16; MnO, 0.12; CuO, 0.033; $Ca(IO_3)_2$, 0.007; CaO, 0.004

Table 2-2. Energy, dry matter, crude protein, ether extract, ash, amino acid, tannin contents and trypsin inhibitor activities in soybean meal, fababeans, peas and experimental diets[z]

	Soybean Meal	Faba-beans	Peas (Ascona)	Peas (Radley)	SBM[y]	SF	AP	RP
Gross energy (MJ kg^{-1})	17.8	17.0	16.4	16.5	16.6	16.6	16.4	16.3
Dry matter (%)	89.3	89.1	87.4	88.3	88.7	88.8	88.8	89.2
Crude protein (%)	45.1	27.4	21.9	21.0	19.5	20.3	15.1	15.1
Ether extract (%)	1.98	0.99	1.51	1.29	3.93	3.71	3.90	3.76
Ash (%)	5.15	2.89	2.91	2.57	6.09	5.84	4.30	4.62
Amino acids (%)								
Indispensable								
Arginine	2.80	2.44	1.87	1.79	1.26	1.53	1.27	1.26
Histidine	1.12	0.68	0.55	0.52	0.49	0.49	0.36	0.37
Isoleucine	1.99	1.14	0.90	0.93	0.90	0.88	0.62	0.65
Leucine	3.28	1.90	1.50	1.56	1.47	1.44	1.02	1.12
Lysine	2.93	1.67	1.60	1.71	1.25	1.23	1.05	1.23
Phenylalanine	2.22	1.08	1.10	1.02	0.96	0.87	0.74	0.70
Threonine	1.65	0.94	0.81	0.82	0.73	0.71	0.55	0.59
Valine	2.05	1.23	0.98	1.02	0.93	0.91	0.67	0.71
Dispensable								
Alanine	1.79	1.06	0.95	0.92	0.84	0.83	0.65	0.65
Aspartic acid	5.21	2.92	2.41	2.41	2.21	2.18	1.60	1.71
Glutamic acid	8.67	4.43	3.80	3.79	3.72	3.52	2.54	2.69
Glycine	1.53	1.11	0.89	0.82	0.71	0.77	0.60	0.59
Serine	2.04	1.22	0.97	0.94	0.93	0.91	0.65	0.68
Tyrosine	1.37	0.81	0.56	0.53	0.59	0.61	0.37	0.38
Tannins[x]	0.08	0.50	0.04	0.04	0.04	0.21	0.03	0.03
TIA[w]	3.00	2.40	1.12	4.60	1.33	1.57	0.76	3.24

[z]as-fed basis. [y]See footnote *z* of Table 2-1. [x]% catechin equivalents. [w]mg trypsin inhibited g^{-1}.

Table 2-3. Effect of diet on volume of pancreatic juice and concentrations and flows of nitrogen and protein and specific and total enzyme activities in pancreatic juice from pigs in Experiments 1 and 2

		Experiment 1			Experiment 2		
Item	Units	SBM[z]	SF	SE[y]	AP	RP	SE[x]
Volume	mL 24 h^{-1}	854	714	89	2029	2221	250
Nitrogen	g L^{-1}	1.64	1.77	0.06	0.60	0.59	0.01
	g 24 h^{-1}	1.40	1.26	0.15	1.20	1.29	0.13
Protein	g L^{-1}	5.51	5.68	0.23	2.12	1.84	0.11
	g 24 h^{-1}	4.71	4.06	0.35	4.30	4.09	0.34
Enzyme activities:							
Amylase	specific[w]	775.5	786.1	57.5	94.1	86.7	9.4
	total 24 h[v]	662.3	561.3	56.8	190.9	192.6	36.9
Lipase	specific[w]	20.1	22.4	3.7	11.3	8.7	1.1
	total 24 h[v]	17.2	16.0	4.4	22.9	19.3	3.1
Chymotrypsin	specific[w]	36.8	37.3	1.9	14.2	13.1	0.6
	total 24 h[v]	31.4	26.6	3.2	28.8	29.1	2.6
Trypsin	specific[w]	41.2*b*	53.7*a*	3.6	24.6	22.8	1.5
	total 24 h[v]	35.2	38.3	4.2	49.9	50.6	5.5

[z]See footnote z of Table 2-1.
[y]Standard error of the mean for Experiment 1; n = 12 except for 24 h means where n = 4.
[x]Standard error of the mean for Experiment 2; n = 15 except for 24 h means where n = 5.
[w]Units L^{-1} x 10^{-3}.
[v]Units 24 h^{-1} x 10^{-3}.
[1]Means in the same row, within the same experiment, followed by a different letter differ at $P < 0.05$.

Table 2-4. Effect of experimental period on volume of pancreatic juice and concentrations and flows of nitrogen and protein and specific and total enzyme activities in pancreatic juice from pigs in Experiment 2

Item	Units	Periods 1	Periods 2	SE[z]
Volume	mL 8 h^{-1}	673	743	47
	mL 24 h^{-1}	2019	2230	250
Nitrogen	g L^{-1}	0.57*b*	0.60*a*	0.01
	g 8 h^{-1}	0.39	0.44	0.03
	g 24 h^{-1}	1.16	1.33	0.13
Protein	g L^{-1}	1.78*b*	2.18*a*	0.11
	g 8 h^{-1}	1.20*b*	1.54*a*	0.09
	g 24 h^{-1}	3.59	4.65	0.34
Enzyme activities:				
Amylase	specific[y]	68.6*b*	112.2*a*	9.4
	total 8 h^{x}	46.2*b*	83.4*a*	6.8
	total 24 h^{w}	138.5	250.2	36.9
Lipase	specific[y]	8.2	11.9	1.1
	total 8 h^{x}	5.5*b*	8.8*a*	0.9
	total 24 h^{w}	16.6	26.5	3.1
Chymotrypsin	specific[y]	12.7	14.6	0.6
	total 8 h^{x}	8.5*b*	10.8*a*	0.6
	total 24 h^{w}	25.6	32.6	2.6
Trypsin	specific[y]	21.7	25.8	1.5
	total 8 h^{x}	14.6*b*	19.2*a*	1.1
	total 24 h^{w}	43.8	57.5	5.5

[z]Standard error of the mean; n = 15 except for 24 h means where n = 5.
[y]Units $L^{-1} \times 10^{-3}$.
[x]Units 8 $h^{-1} \times 10^{-3}$.
[w]Units 24 $h^{-1} \times 10^{-3}$.
Means in the same row followed by a different letter differ at $P < 0.05$.

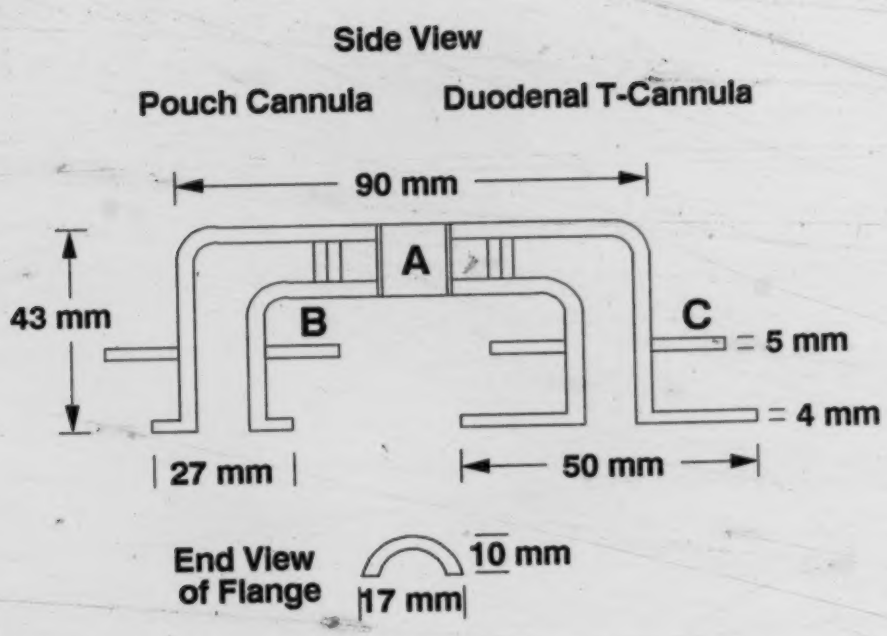

Fig. 2-1. Schematic diagram of a modified pancreatic pouch re-entrant cannula. Where **A** is a one-way valve and **B** and **C** are retaining rings.

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CHAPTER 3

CONCENTRATIONS AND FLOWS OF TOTAL, PROTEIN-BOUND AND FREE AMINO ACIDS IN PANCREATIC JUICE COLLECTED FROM YOUNG PIGS FED DIETS CONTAINING FABABEANS AND PEAS[1]

A. Introduction

The secretions of the exocrine pancreas are required for digestion. These secretions include digestive enzymes, polypeptide enzyme cofactors and inhibitors, mucins, bicarbonate, urea, sodium, potassium and chloride (Kidder and Manners 1978; Schulz 1987; Rinderknecht 1993). The regulation of secretion and activities of the various enzymes have been extensively studied (Solomon 1987; Chey 1993). The activities are highly dependent on diet composition, age of the animals, feeding regimen and time of sampling in relation to time of feeding (Corring et al. 1989; Makkink and Verstegen 1990). However, there is a scarcity of information on amino acid (AA) secretion and composition in pancreatic secretions. Corring and Jung (1972) and Pöhland et al. (1993) determined the AA composition of CP (g AA 16 g N^{-1}) in pancreatic juice from growing pigs, it has also been determined in pancreatic juice from lambs (Hamza 1976). The AA composition and

[1]A version of this chapter has been published. V. M. Gabert, W. C. Sauer, S. Li and M. -Z. Fan. 1996. J. Sci. Food Agric. 70: 256-262.

sequence of many enzymes in pancreatic juice have been determined (e.g., Cozzone et al. 1970; Winkler et al. 1990). However, no investigations have been carried out to determine if the composition of total AA is different from the composition of protein-bound AA and whether or not there are free AA present in pancreatic juice. This information is important for the modeling of endogenous AA flows and will assist in establishing the contribution of pancreatic to endogenous AA in digesta from the distal ileum of pigs (Souffrant 1991; Bastianelli et al. 1996).

The objectives of this study were to determine the contribution of protein-bound and free AA to total AA in pancreatic juice from young pigs and to investigate if the concentration, flow and composition of total, protein-bound and free AA were affected by the type of diet fed, age of the animals and time of sampling.

B. Materials and Methods

Animals and Diets

A detailed description of the experimental procedures including diet formulation and composition, cannula design and protocol for the collection and pooling of pancreatic juice is presented in Chapter 2.

Chemical Analyses

Pancreatic juice was prepared for the determination of free AA in the following manner. Vials containing 1 mL of undiluted pancreatic juice were thawed at 5°C. The vials were placed in a rack in an ice bath and 100 µL of pancreatic juice from each vial was pipetted into 10 x 75 mm disposable borosilicate glass tubes and 300 µL of an internal

standard solution (340 µM ethanolamine hydrochloride and 320 µM DL-β-amino-n-butyric acid, dissolved in distilled and deionized water) was added. The contents were mixed using a Vortex Genie™ (Scientific Industries, Bohemia, NY) and proteins were precipitated by adding 400 µL of 5% (w/v) trichloroacetic acid. The contents were mixed again several times with a Vortex Genie™ and centrifuged at 5°C and 1110 x g for 15 min to pellet the precipitated proteins. Amino acid analyses on 100 µL of the supernatant were initiated after centrifugation.

For the determination of the concentration of total AA in pancreatic juice, samples were thawed as previously described and 500 µL was pipetted into 13 x 100 mm screw-capped test tubes, mixed with 6 mL of 6 M hydrochloric acid, flushed with N_2, capped and hydrolyzed at 110°C for 24 h.

Analyses of free and total AA were carried out by a fluorometric method involving pre-column derivatization with *o*-phthaldialdehyde according to Jones and Gilligan (1983) using the HPLC system described by Dugan et al. (1989). Peaks were recorded and integrated with the EZchrom™ Chromatography Data System (version 2.12, Shimadzu Scientific Instruments Inc., Columbia, MD). Methionine, cysteine, tryptophan and proline were not determined. Protein-bound AA were calculated as the difference between total (mM) and free AA (mM). Due to the conversion of glutamine and asparagine to glutamic and aspartic acid, respectively, during hydrolysis, protein-bound glutamic and aspartic acid were calculated by subtracting free glutamic acid plus free glutamine or free aspartic acid plus free asparagine from total glutamic or aspartic acid, respectively.

The daily flows of total, protein-bound and free AA were calculated by multiplying the concentrations of free, protein-bound and total AA by the volume of pancreatic juice secreted per day (Chapter 2).

Statistical Analyses

Analyses of variance were carried out according to procedures described in Chapter 2. The diets, 8-h sampling periods and experimental periods did not affect ($P > 0.05$) pancreatic secretions of AA. Therefore, the data were pooled within each experiment. Pearson correlation analyses (SAS Institute, Inc. 1988) were used to determine the relationships between the concentrations of protein-bound and free AA in pancreatic juice. Stepwise multiple linear regression procedures (SAS Institute, Inc. 1988) were used to investigate the relationship between specific enzyme activity and protein concentration (Chapter 2) and the concentrations of protein-bound AA, respectively.

C. Results

Diet, 8-h sampling period and experimental period did not affect ($P > 0.05$) the concentrations, flows and composition of total, protein-bound and free AA in pancreatic juice in either experiment. The AA composition (g 16 g N^{-1}) of pancreatic juice is presented in Table 3-1. The AA composition in pancreatic juice from pigs in Experiment 1 was similar to that from pigs in Experiment 2 and those reported by Corring and Jung (1972) and Pöhland et al. (1993).

Concentrations of total, protein-bound and free AA in pancreatic juice from pigs in both experiments are presented in Table 3-2. The concentrations of total, protein-bound and

free AA were higher in pancreatic juice from younger pigs in Experiment 1 than from the older pigs in Experiment 2. This is in agreement with both the protein concentrations and specific enzyme activities which were higher in pancreatic juice from pigs in Experiment 1 than in Experiment 2 (Chapter 2).

Daily flows of total, protein-bound and free AA in pancreatic juice from pigs in both experiments are presented in Table 3-3. The volume of pancreatic juice secreted by the pigs in Experiment 2 was approximately three times greater than that secreted by pigs in Experiment 1 (Chapter 2). Therefore, the flows of total, protein-bound and free AA were similar in both experiments even though the concentrations of total, protein-bound and free AA were higher in Experiment 1 than in Experiment 2.

The percentage of protein-bound AA in pancreatic juice from pigs in both experiments is presented in Table 3-4. For the indispensable AA, the percentages ranged from 43.2 to 80.7% in Experiment 1 and from 45.0 to 81.9% in Experiment 2. For the dispensable AA, the percentages ranged from 57.8 to 92.7% in Experiment 1 and from 57.1 to 90.8% in Experiment 2. The contribution of protein-bound to total AA is quite variable and free AA make a substantial contribution to total AA in pancreatic juice. The overall mean of the percentage of protein-bound AA was 72.6% in Experiment 1 and 74.0% in Experiment 2.

Correlation coefficients between the concentrations of free and protein-bound AA in pancreatic juice are shown in Table 3-5. The coefficients ranged from 0.73 for glutamic acid to 0.89 for valine indicating that the concentrations of free and protein-bound AA are highly correlated.

Equations from multiple linear regression analyses of the relationships between enzyme activities and protein concentration (Chapter 2) and the concentrations of protein-bound AA in pancreatic juice, respectively, are presented in Table 3-6. The highest R^2 values were obtained for chymotrypsin (0.94) and amylase (0.93) activities and protein concentration (0.98). The R^2 for trypsin activity was lower (0.68); the lowest R^2 was obtained for lipase activity (0.44).

D. Discussion

The lack of effect of 8-h sampling period and experimental period on AA composition in pancreatic juice (Table 3-1) is in agreement with results reported by Corring and Jung (1972) and Pöhland et al. (1993). They reported that the AA composition in pancreatic juice was not affected by sampling time or diet. Corring and Jung (1972) concluded that the composition of the mixture of enzymes secreted from the exocrine pancreas does not change because the AA composition remains constant. Nonetheless, during adaptation to diets differing in protein, starch and fat content, i.e., from low to high, the composition would be expected to change. Zymogen granule stores inside acinar cells are replaced within a few hours by granules containing newly synthesized proteins (Scheele and Kern 1993; O'Keefe et al. 1994). However, the composition of the mixture of enzymes did not change even though large differences existed in amylase activity between the experiments; a result of the different starch (amylose and amylopectin) contents of the diets (Chapter 2). In both experiments, pancreatic juice contained relatively high amounts of

aspartic and glutamic acid and slightly more dispensable than indispensable AA which is in agreement with studies by Corring and Jung (1972) and Pöhland et al (1993).

In both experiments, the most abundant AA in pancreatic juice, on a total basis, were aspartic acid, glycine, glutamic acid, valine, leucine, serine and alanine (Table 3-2). In both experiments, there were higher concentrations of dispensable than indispensable total AA in pancreatic juice. The most abundant protein-bound AA in pancreatic juice were aspartic acid, glycine, glutamic acid, serine, valine, alanine and leucine (Table 3-2). The concentrations of these AA are related to the AA composition of the various enzymes in pancreatic juice. Aspartic acid, glutamic acid and valine make a large contribution to the total amount of AA in amylase (Cozzone et al. 1970). The most abundant AA in trypsin are alanine, aspartic acid, glutamic acid, glycine, leucine, serine and valine (Charles et al. 1963). Alanine, aspartic acid, glycine, serine and valine occur in relatively high amounts in α-Chymotrypsin (Charles et al. 1967). The most predominant AA in lipase are aspartic acid, glycine, leucine, serine and valine (Winkler et al. 1990). As was observed for total AA, there were higher concentrations of dispensable than indispensable protein-bound AA in pancreatic juice.

In pancreatic juice from pigs in both experiments, the most abundant free AA were leucine and lysine (Table 3-2). In contrast to the concentrations of total and protein-bound AA, pancreatic juice had higher concentrations of free indispensable than dispensable AA. There is no literature concerning the concentrations of free AA in pancreatic juice. Future investigations are warranted, especially with respect to the very high concentration of leucine which contributed 15.1 and 14.8% to total free AA in pancreatic juice in

Experiments 1 and 2, respectively. Free AA in pancreatic juice may arise from acinar cells, epithelial cells of intralobular and interlobular ducts or epithelial cells of the main pancreatic duct which have a high turnover rate (Kern 1993). The concentration of free AA in pancreatic juice appear to be dependent on the amount of enzymes and other proteins secreted (Table 3-5).

The release of AA from hydrolysis of pancreatic proteins by active trypsin and(or) chymotrypsin and the action of bacterial proteases must be considered and investigated further. Given that the pancreatic pouch is a segment of intestine, it is not sterile and bacterial proteases may be present; it may also secrete a small amount of enterokinase, both of which may activate some trypsinogen and(or) chymotrypsinogen (Moran 1982). For example, trypsin cleaves peptide bonds adjacent to arginine and lysine and these AA are the first released during digestion of proteins (Low 1980; Rinderknecht 1993).

The concentrations of alanine, citrulline, glutamine, isoleucine, serine, threonine and valine fall within the range of those found in porcine plasma which suggests that they may originate from free AA in blood (Keith et al 1977; Pösö and Jensen-Waern 1992). In contrast, the concentrations of asparagine, glutamic acid and glycine are lower and the concentrations of the remaining AA are higher than those found in porcine plasma (Keith et al 1977; Pösö and Jensen-Waern 1992).

The relationships between specific enzyme activities and the concentrations of protein-bound AA (Table 3-6) were not only driven by the AA which are the most prevalent in pancreatic enzymes but also by AA which were less prevalent. The AA which make a lesser contribution to total AA in pancreatic enzymes that remained in the equations were

histidine, threonine, phenylalanine, tyrosine and lysine. This may have been due to co-secretion of other proteins in pancreatic juice such as mucins, co-factors and inhibitors which contain higher amounts of these AA.

Aspartic acid, glycine, glutamic acid and valine contributed the most to total AA flow (Table 3-3). Similarly, aspartic acid, glycine, glutamic acid, valine and serine contributed the most to protein-bound AA flow. In contrast, leucine and lysine contributed the most to free AA flow. For total and protein-bound AA, there were higher daily flows of dispensable than indispensable AA. In contrast, for free AA there were higher daily flows of indispensable than dispensable AA. There is no information in the literature on the daily flows of total, protein-bound and free AA. Further research is needed to assess to which degree these AA are reabsorbed before they reach the distal ileum; this is important in light of increasing interest in understanding which factors affect and contribute to endogenous protein (AA) secretions (Souffrant 1991; Bastianelli et al. 1996).

In conclusion, this study demonstrated that free AA are present in pancreatic juice from young pigs and depending on the AA being considered, the free form makes a large contribution to total AA. The concentrations, flows and compositions of total, protein-bound and free AA in pancreatic juice were not influenced by diet, 8-h sampling period or experimental period. The AA composition of pancreatic juice and the daily flows of total, protein-bound and free AA in pancreatic juice were similar for younger (9.9 kg, Experiment 1) and older (heavier, 22.6 kg, Experiment 2) pigs.

E. Implications

This study demonstrates that free AA are present in pancreatic juice collected from young pigs. However the role and source of free AA have yet to be identified. The AA composition and daily flows of total, protein-bound and free AA were similar for pigs which weighed 9.9 and 22.6 kg and therefore these values could be used as estimates of AA composition and flows in growing and finishing pigs. The AA composition and flows can be used as inputs for characterizing AA fluxes in the digestive tract.

Table 3-1. Amino acid composition of crude protein in pancreatic juice

	Experiment 1[z]	Experiment 2[y]
Amino acids (g 16 g N^{-1}):		
Indispensable		
Arginine	2.6 ± 0.7[x]	2.7 ± 0.7
Histidine	1.3 ± 0.3	1.5 ± 0.4
Isoleucine	3.1 ± 0.7	3.5 ± 0.8
Leucine	4.4 ± 0.9	5.2 ± 1.2
Lysine	2.7 ± 0.7	2.9 ± 0.9
Phenylalanine	2.5 ± 0.6	2.7 ± 0.6
Threonine	3.0 ± 0.7	3.3 ± 0.8
Valine	4.1 ± 0.8	4.9 ± 0.9
Subtotal	23.7	26.7
Dispensable		
Alanine	2.9 ± 0.7	3.3 ± 0.8
Aspartic acid	6.6 ± 1.6	7.2 ± 1.7
Glutamic acid	5.6 ± 1.3	6.8 ± 1.7
Glycine	3.2 ± 0.8	3.8 ± 1.2
Serine	3.4 ± 0.8	3.9 ± 0.9
Tyrosine	3.0 ± 0.7	3.4 ± 0.9
Subtotal	24.7	28.4
Total	48.4 ± 11.2	55.1 ± 13.1

[z]In Experiment 1, n = 24.
[y]In Experiment 2, n = 30.
[x]Standard deviation.

Table 3-2. Concentration of total, protein-bound and free amino acids in pancreatic juice

	Experiment 1[z]			Experiment 2[y]		
Amino acids (mmol L^{-1}):	Total	Protein-Bound	Free	Total	Protein-Bound	Free
Indispensable						
Arginine	1.59 ± 0.47[x]	0.80 ± 0.35	0.79 ± 0.18	0.58 ± 0.17	0.26 ± 0.07	0.32 ± 0.10
Histidine	0.91 ± 0.26	0.57 ± 0.21	0.34 ± 0.10	0.36 ± 0.11	0.26 ± 0.07	0.10 ± 0.04
Isoleucine	2.47 ± 0.69	1.73 ± 0.56	0.74 ± 0.15	0.98 ± 0.27	0.70 ± 0.19	0.28 ± 0.09
Leucine	3.52 ± 0.91	1.85 ± 0.64	1.67 ± 0.38	1.48 ± 0.41	0.84 ± 0.21	0.64 ± 0.21
Lysine	2.00 ± 0.57	0.88 ± 0.33	1.12 ± 0.29	0.74 ± 0.25	0.33 ± 0.09	0.41 ± 0.17
Methionine			0.18 ± 0.05			0.07 ± 0.02
Phenylalanine	1.64 ± 0.48	1.00 ± 0.36	0.64 ± 0.15	0.61 ± 0.17	0.36 ±0.09	0.25 ± 0.08
Threonine	2.69 ± 0.75	2.19 ± 0.68	0.50 ± 0.10	1.02 ± 0.27	0.81 ± 0.22	0.21 ± 0.06
Tryptophan			0.44 ± 0.10			0.16 ± 0.05
Valine	3.71 ± 0.91	2.93 ± 0.75	0.78 ± 0.20	1.56 ± 0.35	1.27 ± 0.27	0.29 ±0.09
Subtotal	18.53	11.95	7.20	7.33	4.83	2.73
Dispensable						
Alanine	3.44 ± 0.94	2.59 ± 0.76	0.85 ± 0.22	1.39 ± 0.39	1.06 ± 0.28	0.33 ± 0.11
Asparagine			0.43 ± 0.10			0.15 ± 0.05
Aspartic acid	5.27 ± 1.56	4.77 ± 1.46[w]	0.07 ± 0.03	2.00 ± 0.58	1.82 ± 0.52[w]	0.03 ± 0.02
Glutamic acid	4.05 ± 1.12	3.23 ± 0.99[v]	0.09 ± 0.03	1.73 ± 0.51	1.38 ± 0.40[v]	0.04 ± 0.02
Glutamine			0.73 ± 0.15			0.31 ± 0.10
Glycine	4.46 ± 1.32	4.15 ± 1.28	0.31 ± 0.06	1.91 ± 0.63	1.72 ± 0.56	0.19 ± 0.07
Serine	3.47 ± 0.96	2.95 ± 0.87	0.52 ± 0.13	1.39 ± 0.40	1.22 ± 0.34	0.17 ± 0.06
Tyrosine	1.77 ± 0.51	1.04 ± 0.39	0.73 ± 0.15	0.70 ± 0.22	0.40 ± 0.13	0.30 ± 0.09
Subtotal	22.46	18.73	3.73	9.12	7.60	1.52
Intermediates						
Citrulline			0.023 ± 0.007			0.010 ± 0.005
Ornithine			0.055 ± 0.021			0.040 ± 0.011
Taurine			0.018 ± 0.007			0.013 ± 0.006

[z]In Experiment 1, n = 24. [y]In Experiment 2, n = 30. [x]Standard deviation. [w]Total aspartic acid - (free asparagine + free aspartic acid). [v]Total glutamic acid - (free glutamine + free glutamic acid).

Table 3-3. Flow of total, protein-bound and free amino acids in pancreatic juice

Amino acids (mmol 24 h^{-1}):	Experiment 1[z]			Experiment 2[y]		
	Total	Protein-Bound	Free	Total	Protein-Bound	Free
Indispensable						
Arginine	1.20 ± 0.38[x]	0.59 ± 0.20	0.61 ± 0.22	1.21 ± 0.52	0.53 ± 0.19	0.68 ± 0.33
Histidine	0.69 ± 0.20	0.43 ± 0.12	0.26 ± 0.11	0.74 ± 0.28	0.53 ± 0.19	0.21 ± 0.09
Isoleucine	1.88 ± 0.57	1.30 ± 0.38	0.58 ± 0.21	2.04 ± 0.74	1.46 ± 0.52	0.58 ± 0.23
Leucine	2.68 ± 0.80	1.38 ± 0.40	1.30 ± 0.46	3.06 ± 0.74	1.73 ± 0.54	1.33 ± 0.53
Lysine	1.51 ± 0.44	0.66 ± 0.22	0.85 ± 0.27	1.54 ± 0.63	0.68 ± 0.21	0.86 ± 0.43
Methionine			0.14 ± 0.05			0.14 ± 0.07
Phenylalanine	1.24 ± 0.38	0.75 ± 0.23	0.49 ± 0.17	1.28 ± 0.46	0.76 ± 0.23	0.52 ± 0.23
Threonine	2.04 ± 0.62	1.64 ± 0.49	0.40 ± 0.14	2.13 ± 0.73	1.70 ± 0.60	0.43 ± 0.14
Tryptophan			0.34 ± 0.14			0.32 ± 0.12
Valine	2.83 ± 0.82	2.23 ± 0.66	0.60 ± 0.19	3.24 ± 1.07	2.65 ± 0.85	0.59 ±0.23
Subtotal	14.07	8.98	5.57	15.24	10.04	5.66
Dispensable						
Alanine	2.61 ± 0.77	1.95 ± 0.57	0.66 ± 0.23	2.89 ± 1.05	2.21 ± 0.77	0.68 ± 0.28
Asparagine			0.34 ± 0.13			0.32 ± 0.14
Aspartic acid	3.99 ± 1.25	3.60 ± 1.12[w]	0.05 ± 0.02	4.18 ± 1.62	3.79 ± 1.44[w]	0.07 ± 0.05
Glutamic acid	3.07 ± 0.92	2.43 ± 0.71[v]	0.07 ± 0.02	3.61 ± 1.47	2.86 ± 1.16[v]	0.10 ± 0.07
Glutamine			0.57 ± 0.20			0.65 ± 0.26
Glycine	3.37 ± 1.02	3.13 ± 0.95	0.24 ± 0.08	4.08 ± 2.15	3.67 ± 1.88	0.41 ± 0.27
Serine	2.64 ± 0.80	2.23 ± 0.66	0.41 ± 0.16	2.88 ± 1.05	2.52 ± 0.92	0.36 ± 0.13
Tyrosine	1.34 ± 0.41	0.77 ± 0.23	0.57 ± 0.20	1.44 ± 0.54	0.82 ± 0.30	0.62 ± 0.25
Subtotal	17.02	14.11	2.91	19.08	15.87	3.21
Intermediates						
Citrulline			0.018 ± 0.006			0.020 ± 0.011
Ornithine			0.039 ± 0.009			0.082 ± 0.027
Taurine			0.013 ± 0.003			0.026 ± 0.008

[z]In Experiment 1, n = 8. [y]In Experiment 2, n = 10. [x]Standard deviation. [w]Total aspartic acid - (free asparagine + free aspartic acid). [v]Total glutamic acid - (free glutamine + free glutamic acid).

Table 3-4. Percentage of protein-bound amino acids in pancreatic juice[z]

	Experiment 1[y]	Experiment 2[x]
Amino acids:		
Indispensable		
Arginine	49.1 ± 8.3[w]	45.0 ± 3.3
Histidine	62.5 ± 7.5	72.3 ± 3.2
Isoleucine	69.4 ± 4.2	71.8 ± 1.9
Leucine	52.0 ± 6.5	57.0 ± 3.2
Lysine	43.2 ± 6.6	45.1 ± 5.0
Phenylalanine	60.1 ± 5.2	60.2 ± 3.1
Threonine	80.7 ± 3.2	79.8 ± 1.6
Valine	78.9 ± 3.1	81.9 ± 1.8
Dispensable		
Alanine	75.0 ± 3.5	76.9 ± 1.5
Aspartic acid	90.3 ± 1.3	90.8 ± 0.8
Glutamic acid	79.3 ± 3.0	79.6 ± 1.4
Glycine	92.7 ± 1.3	90.3 ± 1.0
Serine	84.8 ± 2.7	87.6 ± 0.9
Tyrosine	57.8 ± 5.9	57.1 ± 2.8
Total[v]	72.6 ± 3.9	74.0 ± 1.5

[z](mmol L^{-1} of each protein-bound amino acid/mmol L^{-1} of each total amino acid) x 100%.
[y]In Experiment 1, n = 24.
[x]In Experiment 2, n = 30.
[w]Standard deviation.
[v](mmol L^{-1} sum of protein-bound amino acids/mmol L^{-1} sum of total amino acids) x 100%

Table 3-5. Correlation coefficients between the conentrations of free amino acids and protein-bound amino acids in pancreatic juice[z]

Indispensable	r[y]	*Dispensable*	r[y]
Arginine	0.78	Alanine	0.86
Histidine	0.74	Aspartic acid	0.77
Isoleucine	0.88	Glutamic acid	0.73
Leucine	0.78	Glycine	0.79
Lysine	0.80	Serine	0.85
Phenylalanine	0.88	Tyrosine	0.85
Threonine	0.88		
Valine	0.89		

[z]n = 54.
[y]$P < 0.0001$

Table 3-6. Multiple linear regression analyses of the relationships between enzyme activities and protein concentrations and concentration of protein-bound amino acids in pancreatic juice[z]

	Regression Equations	R^2
Amylase	$Y^y = -44.5 - 1194.9X_{his} + 369.1X_{thr} + 234.4X_{val} + 1987.6X_{phe} - 1062.0X_{leu}$	0.93
Lipase	$Y^y = 9.3 + 28.9X_{tyr} + 11.5X_{val} - 44.5X_{leu} + 35.0X_{lys}$	0.44
Chymotrypsin	$Y^y = -2.7 - 22.5X_{his} + 15.5X_{thr} - 21.5X_{arg} - 13.5X_{tyr} + 22.7X_{val} - 17.1X_{leu}$	0.94
Trypsin	$Y^y = 2.4 + 69.8X_{his} + 12.5X_{gly} - 62.6X_{arg}$	0.68
Protein	$Y^x = -0.4 + 1.8X_{asp} + 2.5X_{ser} - 4.7X_{his} - 1.7X_{arg} + 2.4X_{val}$	0.98

[z]n = 54; concentrations of protein-bound amino acids (X) in mmol L^{-1}.
[y]Specific enzyme activity (U L^{-1} x 10^{-3}) from Chapter 2.
[x]Protein concentration (g L^{-1}) from Chapter 2.

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CHAPTER 4

EXOCRINE PANCREATIC SECRETIONS IN GROWING PIGS FED DIETS CONTAINING FISH OIL, RAPESEED OIL[1] OR COCONUT OIL[2]

A. Introduction

The secretions of the exocrine pancreas are required for digestion and the regulation of secretion of the various enzymes has been extensively studied (Corring et al. 1989; Brannon 1990). Enzyme secretion is dependent on diet composition, age, feeding regimen and time of sampling in relation to time of feeding (Corring and Saucier 1972; Hee et al. 1988). For example, as the amount of dietary fat is increased, lipase secretion increases (Mourot and Corring 1979). A pre-translational regulatory mechanism appears to be responsible for increased lipase synthesis irrespective of the degree of saturation of dietary fat (Ricketts and Brannon 1994). In several studies, the rat has been used as a model to study the effect of fatty acid composition on enzyme synthesis and secretion. Inclusion of polyunsaturated fatty acids in the diet increases lipase activity in pancreatic homogenate (Deschodt-Lanckman et al. 1971; Ricketts and Brannon 1994). However, in

[1]Extracted from "double-low" Danish rapeseed (low levels of erucic acid and glucosinolates).

[2]A version of this chapter has been published. V. M. Gabert, M. S. Jensen, H. Jørgensen, R. M. Engberg and S. K. Jensen. 1996. J. Nutr. 126: 2076-2082.

experiments with rats fed diets containing 45% fat, lipase and colipase activities were not affected by degree of saturation or chain length of fatty acids (Saraux et al. 1982).

With the exception of studies by Simoes-Nunes (1986), the pig has not been used as a model to study the effect of fatty acid composition on exocrine pancreatic secretions. Simoes-Nunes (1986) reported that pigs fed diets containing 21% sunflower oil had higher lipase activity in pancreatic homogenate than pigs fed diets with the same level of animal fat. The quality of dietary fat has been shown to affect pancreatic secretions. Ozimek et al. (1995) observed that total activity of lipase in pancreatic juice increased, compared to a diet containing 15% canola oil, when 15% peroxidized canola oil was fed to growing pigs. In pigs, there is a scarcity of information on the effect of fatty acid composition on pancreatic secretion of lipase as well as colipase, an essential cofactor for lipase activation. In addition, there is no information on the effect of the amount or source of dietary fat on the activity of carboxyl ester hydrolase, an enzyme that hydrolyses a variety of lipid substrates (Rinderknecht 1993).

The objective of this study was to obtain further information on the influence of fatty acid composition of fish oil, rapeseed oil and coconut oil, on exocrine pancreatic secretions in growing pigs. Growing pigs were fitted with either a pancreatic pouch re-entrant cannula (Pouch Method, Experiment 1) or a catheter into the pancreatic duct (Catheter Method, Experiment 2). A comparison of these two commonly used methods is presented in Chapter 5.

B. Materials and Methods

Animals and Diets

Two experiments with a total of six crossbred barrows (Danish Landrace x Yorkshire), obtained from the Danish Institute of Animal Science swine herd, were performed. Due to the very intensive nature of the studies, the experiments were carried out in sequence. The average initial BW of the pigs used in these studies was 34.6 kg. The pigs were housed individually in pens and had free access to water and a 16% CP commercial grower diet prior to the experiment.

In both experiments, the pigs were fed three 17% CP diets that contained wheat starch (40.3%), fish meal (22.3%), wheat bran (10.0%) and either 15% fish oil, rapeseed oil or coconut oil. To possibly increase the palatablility of the diets, 10% sucrose was included. Chromic oxide (0.2%) was included in the diets as a marker to determine the digestibilities of the parameters measured. Vitamins and minerals were supplemented to meet or exceed Danish standards for growing pigs (Andersen and Just 1983). The diets were stored in a 5°C walk-in fridge. The chemical composition of the diets is presented in Table 4-1. The pigs were fed 1.65 kg d^{-1} in three meals of equal amounts at 0800, 1600 and 2400 h.

Experiment 1

Three barrows, average initial BW 36.8 kg, were fitted with a pancreatic pouch re-entrant cannula for collection and subsequent return of pancreatic juice (Pouch Method) according to procedures adapted from Hee et al. (1985) and those described in Chapter 2. The construction of the cannulas and surgical procedures are described in Chapter 5.

The experiment was carried out according to a 3 x 3 Latin square design. The average BW of the pigs were 38.8 kg at the start and 53.9 kg at the conclusion of the experiment. Each experimental period consisted of 7 d: 5-d adaptation to the experimental diets followed by a 2-d collection of pancreatic juice. A 5-d adaptation period was selected based on the review of Corring et al. (1989) and the results of Partridge et al. (1982) which indicate that this is a long enough period to allow adaptation to a change in diet.

Pancreatic juice was collected for a total of 24 h, continuously from 1600 to 2400 h on d 6, from 0800 to 1600 h on d 7 and from 2400 h on d 7 to 0800 h on d 8 after which the next period started. One hour before the start of the 2-d collection, the pigs were placed into stainless steel metabolic crates. At the start of each 8 h-sampling period, the cannulas were disconnected and pancreatic juice was collected via Tygon® tubing by free drainage into a polyethylene bottle kept in ice. After each hour of collection, the pH was measured (Model 691, Metrohm, Herisau, Switzerland) and the volume was recorded. Three proportionally pooled samples were prepared by pipetting 5% of the total volume collected during each hour into a polyethylene bottle kept in ice until the end of each 8 h-sampling period. The samples were frozen at -80°C following each 8-h sampling period. The subsample was replaced with 154 mM sodium chloride and pancreatic juice was continuously reinfused into the duodenal T-cannula from a second bottle with a peristaltic pump (Model 2650, Ole Dich Instrument Makers, Hvidovre, Denmark). The pump was adjusted frequently so that the rate of pancreatic juice return to the duodenum was as close as possible to the rate of secretion. Pancreatic juice from the last hour of collection

of the most recent 8-h sampling period was infused during the first hour of the next collection.

Feces were collected during pancreatic juice collection and frozen at -25°C immediately after collection.

Experiment 2

Three barrows, average initial BW 32.4 kg, were fitted with a catheter into the pancreatic duct and a simple T-cannula inserted into the duodenum for collection and subsequent return of pancreatic juice (Catheter Method) according to procedures described by Wass (1965), Pierzynowski et al. (1988) and Thaela et al. (1995). The preparation of the catheters and surgical procedures are described in Chapter 5.

The average BW of the pigs at the start and at the conclusion of the experiment were 39.4 and 53.6 kg, respectively. The experiment was carried out according to a 3 x 3 Latin square design. Length of experimental period, collection times, sampling and reinfusion procedures were identical to those described for experiment one.

Chemical and Enzymatic Analyses

Feces were freeze-dried, pooled within pig and ground with a mortar and pestle. Dry matter determination was carried out according to Association of Official Analytical Chemists (1990) methods. Crude protein was measured by the Kjeldahl method using a Kjell-Foss 16200 AutoAnalyser (Foss Electric, Hillerød, Denmark). Gross energy was determined with an IKA-C 400 Bomb Calorimeter (IKA Werke, Janke & Kunkel, Staufen, Germany). Starch plus free sugars were measured according to procedures described by Christensen (1980). Chromic oxide was measured using the method of

Schürch et al. (1950). Fat was extracted with diethyl ether after acid-hydrolysis (Stoldt 1952). Determination of fatty acids in Stoldt fat extract was carried out as described by Engberg et al. (1993).

The concentration of protein in pancreatic juice was measured according to the method of Lowry et al. (1951), modified to be performed in a 96-microwell plate, using bovine serum albumin (code A7638, Sigma Chemical, St. Louis, MO) as a standard.

Bicarbonate concentration in pancreatic juice was determined by diluting 1 mL of pancreatic juice with 9 mL of distilled, deionized water and autotitrating to pH 4 with 20 mM hydrochloric acid. It was decided to titrate to pH 4, two pH units below the pK_a of the conversion of bicarbonate to carbonic acid, to ensure complete protonation of bicarbonate.

A detailed summary of procedures used in each enzymatic assay is presented in Table 8-1 (Chapter 8). Amylase activity was measured using the Phadebas® amylase reagent as a substrate (Pharmacia Diagnostics, Uppsala, Sweden). Carboxyl ester hydrolase (CEH) activity was determined according to the method of Erlanson (1970) and modified to be performed in a 96-microwell plate.

Activation of trypsinogen to trypsin, was carried out according to the method of Glazer and Steer (1977) with some modifications. Pancreatic juice was diluted 50 times in a buffer [50 mM tris(hydroxymethyl)-aminomethane (code 8382, Merck, Darmstadt, Germany), 50 mM calcium chloride and 1 g L^{-1} bovine serum albumin (code A4378, Sigma Chemical) and pH 8.1]. Subsequently, 50 µL of an enterokinase solution [50 mg porcine enteropeptidase (code E0632, Sigma Chemical) dissolved in 50 mL of 154 mM

sodium chloride] was added to 1 mL of diluted pancreatic juice and incubated at 30°C for 20 min. Trypsin activity was measured according to procedures described by Erlanger et al. (1961) modified to be performed in a 96-microwell plate.

Activation of chymotrypsinogen to chymotrypsin was performed using the procedure described by Glazer and Steer (1977) with some modifications. Pancreatic juice was diluted 50 times in a buffer [100 mM tris(hydroxymethyl)-aminomethane, 10 mM calcium chloride, 1 g L^{-1} bovine serum albumin and pH 7.8]. Thereafter, 30 µL of a trypsin solution [10 mg bovine trypsin (code T8253, Sigma Chemical) dissolved in 10 mL of 1 mM hydrochloric acid] was added to 1 mL of diluted pancreatic juice and the solution was incubated at 4°C for 3 h. The methods of DelMar et al. (1979) and Lainé et al. (1993) were used to determine chymotrypsin activity and modified to be performed in a 96-microwell plate.

Lipase activity was measured according to the titrimetric method of Erlanson-Albertsson et al. (1987) in the presence of bile salts and excess colipase. The activity of colipase, an essential cofactor for lipase (Rinderknecht 1993), was determined as the amount of lipase activity dependant on the presence of colipase; the lipase assay was performed without adding colipase prior to titration (Borgström and Hildebrand 1975).

Enzyme activity in pancreatic juice was expressed as units (U) per liter (specific activity) and per 8 or 24 h (total activities). One U of enzyme activity was defined as the hydrolysis of 1 µmol of substrate in 1 min. Total enzyme activities were calculated as specific activity x volume of pancreatic juice secreted per 8 or 24 h.

Statistical Analyses

Analyses of variance was carried out according to a 3 x 3 Latin square design (Steel and Torrie 1980) with period, pig and diet as main effects using the GLM Procedure of the SAS Institute, Inc. (1988). Volume and pH of pancreatic juice, bicarbonate and protein concentration and secretion as well as specific and total enzyme activities during the three 8-h sampling periods were analyzed as repeated measures (Gill and Hafs 1971) using the GLM Procedure of the SAS Institute, Inc. (1988). However, test for sphericity ($P > 0.05$) for some parameters indicated that F values from split-plot analyses were valid (SAS Institute, Inc. 1991). The error term period x pig x diet was used to test the effects of period, pig and diet in the whole plot. Time (8-h sampling period) and diet x time were included in the split-plot. Where appropriate, means were compared using the Student-Newman-Keuls' multiple range test (Steel and Torrie 1980).

C. Results

The pigs remained healthy and consumed their meal allowances throughout each experiment. Postmortem examinations, carried out at the conclusion of each experiment, revealed no intestinal adhesions or other abnormalities.

Experiment 1

The volume of pancreatic juice, pH and protein and bicarbonate secretions (Table 4-2) were not affected ($P > 0.05$) by the inclusion of fish oil, rapeseed oil or coconut oil in the diets. The specific and total activities of trypsin, chymotrypsin, CEH, lipase, colipase and amylase were also not affected ($P > 0.05$) by the experimental diets. There

were no differences ($P > 0.05$) in the volume of pancreatic juice, pH, protein and bicarbonate secretions and specific and total enzyme activities between the experimental periods or 8-h sampling periods. Therefore, 24-h means are presented for total activities.

Apparent digestibilities of gross energy, DM, CP and Stoldt fat did not differ ($P > 0.05$) between the experimental diets (Table 4-3). There was no effect ($P > 0.05$) of experimental period on the digestibilities of the parameters measured.

Experiment 2

Inclusion of fish oil, rapeseed oil or coconut oil in the diets did not affect ($P > 0.05$) the volume of pancreatic juice, pH, protein and bicarbonate secretions (Table 4-2). The specific activities of trypsin and CEH were increased ($P < 0.05$) in pancreatic juice from pigs fed the fish oil diet than the rapeseed or coconut oil diets. In contrast, specific chymtrypsin activity was lower ($P < 0.05$) in pancreatic juice from pigs fed the fish oil diet than the rapeseed or coconut oil diets.

The total activity of chymotrypsin was lower ($P < 0.05$) in pancreatic juice from pigs fed the fish oil than the coconut oil diet. The total activity of CEH was higher ($P < 0.05$) in pancreatic juice from pigs fed the fish oil diet than those fed the rapeseed or coconut oil diets. Experimental period or 8-h-sampling period did not affect ($P > 0.05$) volume of pancreatic juice, pH, protein secretion, bicarbonate secretion or specific and total enzyme activities. Therefore, total activities over 24 h are shown.

There was no effect ($P > 0.05$) of experimental diet on apparent digestibilities of gross energy, DM, CP and Stoldt fat (Table 4-3). Furthermore, experimental period did not affect ($P > 0.05$) the digestibilities of the parameters measured.

D. Discussion

The lack of effect of fatty acid composition (Table 4-1) on pancreatic secretions, lipase activity in particular (Table 4-2), is not in agreement with the observations of Simoes-Nunes (1986). In both experiments in this study, long chain and polyunsaturated fatty acids in fish oil or rapeseed oil (Table 4-1) did not increase the specific or total activities of lipase. The level of fat in the diets in this study was chosen on the basis of previous experiments which showed a maximal effect of fatty acid composition on lipase activity when the diet contained 15-20% fat (Simoes-Nunes 1986; Ricketts and Brannon 1994). Considering the limited information, further experiments with different levels and sources of fat should be carried out with pigs.

In the rat, when the level of polyunsaturated fatty acids in the diet is increased, lipase activity in pancreatic homogenate increases (Deschodt-Lanckman et al. 1971; Ricketts and Brannon 1994). Recent research in rats suggests that the degree of saturation of dietary fat may act through a translational mechanism: moderate levels of saturated fat may reduce the efficiency of translation or decrease the synthesis of lipase through a pre-translational mechanism (Ricketts and Brannon 1994). Whether or not the same mechanism exists in the pig remains to be determined.

Luminal fatty acids stimulate pancreatic secretion most likely through a secretin-mediated mechanism (Brannon 1990). However, the effect of chain length and degree of saturation on the volume of pancreatic juice remains to be investigated. Chain length and degree of saturation have been suggested to regulate pancreatic lipase at translational or post-translational levels (Ricketts and Brannon 1994) resulting in different lipase

concentrations in pancreatic tissue. Whether these mechanisms of regulation are independant remains to be elucidated.

The significant effects of the type of fat on specific activities of trypsin, chymotrypsin and CEH and on total activities of chymotrypsin and CEH, when the Catheter Method was used, are difficult to explain. However, the effects of the experimental diets on specific enzyme activities are of minor importance because total enzyme activity reflects the quantity of enzymes that can participate in digestion. Similar results were found in studies with rats fed high fat diets (50%); chymotrypsin activity in pancreatic homogenate was increased when tristearin and arachid oil were fed (Deschodt-Lanckman et al. 1971). In this study, pigs fed the coconut oil diet had a higher total activity of chymotrypsin in pancreatic juice (Table 4-2). However, an increase in chymotrypsin activity was not observed in previous studies with rats or pigs fed diets containing either 17.4 or 21% lard (Simoes-Nunes 1986; Ricketts and Brannon 1994). The higher ($P < 0.05$) total activities of CEH in pancreatic juice from pigs fed the fish oil diet may have been due to an effect of the degree of saturation and chain length of dietary fat on the translational regulatory mechanism of CEH synthesis (Huang and Hui 1991). Fish oil may have a stimulatory effect while rapeseed and coconut oil may have an inhibitory effect.

The digestibilities of fat observed in this study (Table 4-3) are similar to those reported by Jørgensen et al. (1992) for pigs fed diets containing a similar level of animal fat or rapeseed oil.

In conclusion, the results of this study indicate that exocrine pancreatic secretion of lipase and colipase are not affected by fatty acid composition when 15% fish, rapeseed or coconut oil are included in the diet. The total activity of CEH is increased when pigs prepared with the Catheter Method are fed a diet containing 15% fish oil.

E. Implications

The results of this study demonstrate that the adaptation of exocrine pancreatic secretions to dietary fat source may depend on the method used to collect pancreatic juice. When the Catheter Method was used, there were significant effects on CEH secretion which may indicate that this enzyme is involved in the adaptation of exocrine pancreatic secretions to changes in dietary fat source. This is the first study in which CEH activity has been measured in pancreatic juice from growing pigs and this enzyme may have an important role in fat digestion. The similar apparent fecal digestibilities of fat, regardless of which collection method was used, suggest that the use of either method does not impair fat digestion. In addition, the high fat digestibilities indicate that most of the added fat was digested and absorbed and demonstrate that pigs have a relatively large capacity to digest fat.

Table 4-1. Chemical composition of the experimental diets[z,y]

Item	Diets		
	Fish oil	Rapeseed oil	Coconut oil
Gross energy (MJ kg^{-1})	20.4	20.3	20.1
Dry matter (%)	93.3	93.2	93.1
Starch plus free sugars (%)	47.3	46.2	47.2
Crude protein (%)	16.8	16.8	16.9
Stoldt fat (%)	18.8	19.0	18.6
Total fatty acids (%)	14.7	16.7	17.2
Fatty acids (%)[x]			
8:0	--	--	7.7
10:0	--	--	4.9
12:0	--	--	38.9
14:0	7.0	1.0	15.7
16:0	18.9	7.2	10.3
16:1 (*n*-7)	5.4	--	--
18:0	2.1	2.1	2.5
18:1	21.3	51.4	7.6
18:2	4.6	20.1	3.4
18:3 (*n*-3)	2.3	7.9	--
20:1 (*n*-9)	5.7	2.2	1.1
20:5 (*n*-3)	7.4	1.2	1.2
22:1	8.8	1.6	1.5
22:6 (*n*-3)	12.0	2.2	2.1
24:1	1.0	--	--

[z]As-fed basis.

[y]The vitamin-mineral premix provides the following per kilogram of diet: vitamin A, 5000 IU; vitamin D_3, 1000 IU; vitamin E, 60 IU; vitamin K, 2.2 mg; niacin, 22 mg; d-pantothenic acid, 11 mg; riboflavin, 4 mg; pyridoxine, 3.3 mg; thiamin, 2.2 mg; d-biotin, 0.55 mg; vitamin B_{12}, 0.022 mg; Fe, 50 mg; Cu, 20 mg; Zn, 80 mg; Mn, 28 mg; Se, 0.3 mg; I 0.2 mg.

[x]Fatty acids as a percent of total fatty acids; fatty acid concentrations <1% are not included.

Table 4-2. Effect of diet composition on volume, pH and concentrations and flows of bicarbonate and protein and specific and total enzyme activities in pancreatic juice from pigs prepared with the Pouch or Catheter Method

	Pouch Method				Catheter Method			
	Diets				Diets			
Item	Fish oil	Rapeseed oil	Coconut oil	SE[z]	Fish oil	Rapeseed oil	Coconut oil	SE[z]
Volume (mL 24 h^{-1})	2723	2783	2377	375	3279	4138	4848	464
Bicarbonate (mmol 24 h^{-1})	363.3	397.4	285.0	46.9	406.5	526.5	678.7	78.8
Protein (g 24 h^{-1})	19.6	18.9	16.4	1.09	15.5	13.3	14.5	3.77
pH	8.36	8.34	8.34	0.04	8.49	8.49	8.51	0.01
Specific enzyme activities								
Trypsin (U L^{-1})	1059.0	1029.4	1004.2	130.8	6193.9*a*	2658.3*b*	2195.0*b*	290.3
Chymotrypsin (U L^{-1})	2310.1	2300.0	1989.9	100.7	1211.7*b*	2448.0*a*	3377.0*a*	318.0
CEH[y] (U L^{-1})	88.9	94.8	85.7	8.2	931.2*a*	578.7*b*	568.2*b*	44.7
Lipase (U L^{-1} x 10^{-3})	2698.7	2510.4	1927.0	150.9	1626.9	1286.9	1433.6	163.2
Colipase (U L^{-1} x 10^{-3})	326.2	199.2	125.4	74.9	733.7	538.0	707.4	147.1
Amylase (U L^{-1} x 10^{-3})	1401.1	1282.2	1141.1	59.6	469.2	404.0	435.9	38.8
Total enzyme activities								
Trypsin (U 24 h^{-1})	2764.0	2821.0	2331.0	227.0	17708.0	10848.0	9701.0	3291.2
Chymotrypsin (U 24 h^{-1})	5773.7	6285.0	4718.0	309.5	3586.0*b*	9524.0*ab*	15728.0*a*	1042.1
CEH[y] (U 24 h^{-1})	252.7	277.7	189.3	35.0	2964.7*a*	2090.3*b*	2293.0*b*	63.1
Lipase (U 24 h^{-1} x 10^{-3})	6998.3	6928.3	4582.7	706.7	4771.0	4765.0	6432.0	989.4
Colipase (U 24 h^{-1} x 10^{-3})	755.0	528.7	287.0	303.3	2041.0	2026.0	3149.0	828.8
Amylase (U 24 h^{-1} x 10^{-3})	3509.0	3512.3	2693.0	367.9	1374.7	1639.0	2179.0	324.8

[z]Standard error of the mean, n = 3, except for specific enzyme activities where n = 9. In any row within collection method, values not sharing a common superscript are significantly different ($P < 0.05$). [y]Carboxyl ester hydrolase.

Table 4-3. Apparent digestibilities (%) of gross energy, dry matter, crude protein and Stoldt fat in pigs prepared with the Pouch or Catheter Method and fed the experimental diets

	Pouch Method				Catheter Method			
	Diets				Diets			
Item	Fish oil	Rapeseed oil	Coconut oil	SE[z]	Fish oil	Rapeseed oil	Coconut oil	SE[z]
Gross energy	89.4	90.2	87.3	1.27	86.2	87.8	88.9	1.15
Dry matter	89.6	89.8	87.8	0.90	87.2	88.0	89.0	1.05
Crude protein	89.2	89.2	87.6	1.57	83.2	86.1	86.6	1.32
Stoldt fat	83.5	85.6	78.1	2.85	79.1	82.1	82.0	2.74

[z]Standard error of the mean, n = 3.

F. References

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CHAPTER 5

COLLECTION OF PANCREATIC JUICE FROM GROWING PIGS: A COMPARISON OF THE POUCH AND CATHETER METHODS

A. Introduction

To study exocrine pancreatic function and the effect of various dietary factors, hormones, agonists and inhibitors *in vivo*, surgical intervention is necessary to obtain pancreatic juice. Two commonly used methods are employed to collect pancreatic juice in pigs. One method, referred to as the Catheter Method (CM), involves surgical placement of a catheter into the pancreatic duct and insertion of a simple T-cannula into the duodenum for return of collected pancreatic juice (Wass et al. 1965; Corring et al. 1972; Pierzynowski et al. 1988; Thaela et al. 1995). The second method, referred to as the Pouch Method (PM) also involves the permanent re-entrant diversion of pancreatic juice. However, pancreatic juice is collected from an isolated duodenal pouch into which the pancreatic duct enters (Hee et al. 1985; Li 1996; Chapter 2). A variation of this method, which involves connecting the cannula in the duodenal pouch to a re-entrant duodenal cannula has also been used (e.g., Zebrowska et al. 1983; Zebrowska and Low 1987). Various studies have been performed, using either method, to investigate the effects of different levels and sources of dietary fat, protein, starch and fiber on pancreatic secretions (Partridge et al. 1982; Hee 1984; Valette et al. 1992; Li 1996). However, no attention has been paid to compare these methods. An implicit assumption is that pancreatic secretion responds to

changes in the amount and type of dietary nutrients in the same way irrespective which surgical technique is used to prepare the pigs. It is also assumed that the regulatory mechanisms of pancreatic secretions are maintained and not affected by either method. However, severing the duodenum to create an isolated pouch and insertion of a re-entrant cannula may affect the regulation of exocrine pancreatic secretions (Pierzynowski et al. 1988). The presence of a catheter in the pancreatic duct may also affect secretion. Whether or not these two techniques will give similar relative responses under the same experimental conditions remains to be determined.

The objective of this study was to compare the two principal methods used for collecting pancreatic juice. Growing pigs, with approximately the same BW, were surgically fitted with either a pancreatic pouch re-entrant cannula or a catheter in the pancreatic duct.

B. Materials and Methods

Animals

Six crossbred barrows (Danish Landrace x Yorkshire), average initial BW 34.6 kg, were obtained from the Danish Institute of Animal Science swine herd. The pigs were kept individually in stainless steel metabolic crates. The study was conducted in two parts (in sequence). In the first part of the study, the Pouch Method was used to prepare three pigs for complete collection and subsequent return of pancreatic juice. In the second part of the study, three pigs were prepared for the collection and return of pancreatic juice using the Catheter Method. One week separated the completion of the first and the beginning of the second part of the study.

Surgical Procedure

Pouch method. Three pigs were fitted with a pancreatic pouch re-entrant cannula for collection and subsequent return of pancreatic juice according to procedures adapted from Hee et al. (1985) which were presented in Chapter 2. The average initial BW of the PM pigs was 36.8 kg. The procedure involved the isolation of the segment of duodenum in which the pancreatic duct entered and the placement of a simple T-cannula. After the pouch was formed, the two remaining two ends of the duodenum were connected by anastomosis and another simple T-cannula was placed in the duodenum to return pancreatic juice.

The cannulas, shown in Fig. 2-1, were prepared according to procedures presented in Chapter 2. The cannulas were made larger than those used in Chapter 2 which allowed them to be used in growing pigs. The flange of the pouch cannula was 55 mm in length and the distance between the flange and the elbow was 45 mm. The inside diameter (i.d.) of the barrel was 8 mm. The retaining rings were 50 mm in diameter. The cannulas were connected with a plastic one-way valve (Nalgene™ 6120-0010 high density polyethylene check valve, Nalge, Rochester, NY). The retaining rings and the one-way valve were secured by four stainless steel gear clamps (5.6 to 15.9 mm, Tridon, Nashville, TN).

The pigs were sedated with azaperone (Stresnil™, Jansen Pharmaceutica, Beerse, Belgium) and midazolam (Dormicum, Hoffman-La Roche, Basel, Switzerland). Anaesthesia was induced with metomidate hydrochloride (Hypnodil™, Jansen Pharmaceutica). The pigs were intubated and brought under general anaesthesia using a mixture of halothane (Halothane Laboratories, North Augusta, SC) and O_2 and N_2O in a ratio of 2:3.

A 15-cm midline incision was made starting 2 cm caudal to the sternum. The pouch T-cannula was inserted into the duodenum and the duodenum transected approximately 1 cm from each end of the T-cannula. Duodenal end-to-end anastomosis was performed using 4-0 suture (Dexon II Bi-colour, Cyanamid, Gossport, Hampshire, UK). The duodenal T-cannula was inserted 4 cm posterior to the site of anastomosis. The pouch cannula was exteriorized between the second and third last rib; the duodenal T-cannula was exteriorized behind the last rib.

To alleviate pain, Petidin (Nycomed DAK, Roskilde, Denmark) and buprenorphin (Temgesic, Reckitt & Colman, Hull, UK) were administered after surgery. The average final BW of the PM pigs was 53.9 kg.

Catheter method. A catheter was placed into the pancreatic duct in three pigs and a simple T-cannula was inserted into the duodenum for collection and subsequent return of pancreatic juice according to procedures described by Wass (1965), Pierzynowski et al. (1988) and Thaela et al. (1995) with some modifications. The average initial BW of the CM pigs was 32.4 kg. The design of the catheter was adapted from the one used by Pierzynowski et al. (1988) and Thaela et al. (1995). The catheters [outside diameter (o.d.) 2.41 mm and i.d. 1.57 mm] were 20 cm in length and were prepared from Silastic® tubing (Dow Corning, Midland, MI). Two Silastic® cuffs were made by wrapping a thin band of silicone glue around the catheter 4.75 and 10.50 cm from the tip and a movable Silastic® ring (o.d. 15 mm) was placed between these cuffs. In addition, a small band of Silastic® tubing (2 mm in length, o.d. 2.41 mm and i.d. 1.57 mm) was slid onto the catheter 1 cm from the tip and used to help secure the catheter in the pancreatic duct.

The barrel of the T-cannula (o.d. 3.18 mm and i.d. 1.98 mm) was 14 cm in length and cuffs made from silicone glue were wrapped around the barrel at 2.75 and 7.00 cm from the flange of the cannula. A moveable Silastic® ring (o.d. 15 mm) was placed between these cuffs. The non-perforated flange (o.d. 4.88 mm and i.d. 2.64 mm) was 4 cm long and made from Silastic® tubing. The barrel was attached to the flange with silicone glue.

The sedatives and anaesthetics used to prepare the CM pigs and the analgesics that were used after surgery were the same as those used for the PM pigs.

Following surgery, an elastic body net (Bend-a-rete, Pic indolor, Como, Italy) was placed around the mid-section (between the front and hind legs) of the pigs. Two pieces of polystyrene foam (12 cm long, 5 cm wide, 2.5 cm thick) were placed on each side of the incision to prevent the catheter and cannula from kinking when the pig laid on its right side. The average final BW of the pigs was 53.6 kg.

Experimental Procedure

The same length of experimental period, diets, collection times, sampling and re-infusion procedures were used for PM and CM pigs and are described in Chapter 4. The samples used in this study were collected from the pigs used in the previous study (Chapter 4). Between the 2-d collections, the catheters were checked frequently to ensure that they were functioning. The pancreatic pouch re-entrant cannula was disconnected every other day and saline was used to flush out the pouch and the one-way valve. For PM pigs, Apoderm® (a zinc oxide and lanolin-based cream, DAK Laboratory, Copenhagen, Denmark) was liberally applied around the cannulas after washing to minimize skin irritation.

Chemical Analyses and Enzyme Activities

The protein and bicarbonate concentrations and the activities of amylase, carboxyl ester hydrolase, lipase, colipase, trypsin and chymotrypsin were measured according to procedures outlined in Chapter 4. Two aliquots of the same sample of pancreatic juice from PM and CM pigs were either activated or not and trypsin and chymotrypsin activities were determined.

Statistical Analyses

The hourly secretion rates in PM and CM pigs were subjected to analyses of variance (ANOVA) for each hourly collection interval of the 24-h collection period. The GLM Procedure of the SAS Institute, Inc. (1988) was used and the sources of variation were period, pig and diet. In order to compare the hourly volume of secretion and pH of pancreatic juice from PM and CM pigs, the data were pooled within method and a one-way ANOVA was conducted with collection method as source of variation. To compare the Pouch and Catheter Methods, the daily volume of pancreatic juice secreted, protein and bicarbonate secretion and specific and total enzyme activities were pooled within method and a one-way ANOVA was carried out with collection method as the source of variation.

To analyze the effect of activation on the specific and total activities of trypsin and chymotrypsin in pancreatic juice from PM pigs, a one-way ANOVA was conducted. Almost no chymotrypsin (< 1 U L^{-1}) and no trypsin activities were detected in nonactivated pancreatic juice from CM pigs. The specific and total activities of chymotrypsin and trypsin in activated pancreatic juice from CM and PM (with and without activation) pigs were

included in a one-way ANOVA. Where appropriate, treatment means were compared using the Student-Newman-Keuls' multiple range test (Steel and Torrie 1980).

Pearson correlation (SAS Institute, Inc. 1988) was used to evaluate the relationship between specific enzymatic activities and protein concentration, lipase and colipase activities and trypsin and chymotrypsin activities in pancreatic juice from PM and CM pigs.

C. Results

The pigs remained healthy throughout the experiment and consumed their meal allowances. Postmortem visual examination was carried out after each part of the study. The pancreas from each pig appeared normal. No fistulas were formed between the duodenal pouch and surrounding intestine in PM pigs.

The diurnal pattern of secretion of pancreatic juice observed in pigs prepared with the Pouch or Catheter Method is presented in Fig. 5-1. The secretion rate in CM pigs was greater ($P < 0.05$) than in PM pigs at several instances throughout the collection. The secretion rate of pancreatic juice in CM pigs was more variable than in PM pigs, which is shown by the greater standard deviations of the mean volume secreted during each interval. The large amount of variation is a direct result of the large difference in the hourly volume of pancreatic juice secreted between the pigs and the absence of a regular pattern of secretion. A similar observation was made by Partridge et al. (1982). In contrast, a distinct pattern, a peak right after feeding in the volume of pancreatic juice secreted, was observed in some other studies (e.g., Pierzynowski et al. 1988; Thaela et al. 1995).

The diurnal change in the pH of pancreatic juice from PM and CM pigs is also presented in Fig. 5-1. For each hourly interval, the pH of pancreatic juice from CM pigs was higher ($P < 0.01$) than in pancreatic juice from PM pigs.

The diurnal pattern of secretion of pancreatic juice in PM pigs (Pigs A, B and C) during each experimental period is shown in Fig. 5-2. The hourly secretion rates did not differ between pigs ($P > 0.05$) or experimental periods ($P > 0.05$). The hourly rates of secretion of pancreatic juice in CM pigs (Pigs D, E and F) in each experimental period are also shown in Fig. 5-2. When the hourly secretion rates from each CM pig were used in statistical analyses, a pig effect ($P < 0.05$) was observed from 2200 to 2300 h, 0100 to 0200 h, 0400 to 0500 h and 0500 to 0600 h.

Daily volume of secretion, concentration and daily output of protein and bicarbonate and specific and total enzyme activities in pancreatic juice from PM and CM pigs are presented in Fig. 5-3. Pigs prepared with the Catheter Method secreted more ($P < 0.05$) pancreatic juice, 4.1 versus 2.6 L 24 h^{-1}, than PM pigs. The level of protein in pancreatic juice was considerably higher ($P < 0.001$) for PM compared to CM pigs, 7.21 versus 4.08 g L^{-1}, respectively. However, total daily protein secretion did not differ ($P > 0.05$) between PM and CM pigs. The concentration of bicarbonate in pancreatic juice did not differ ($P > 0.05$) between PM and CM pigs.

The specific and total activities of amylase in pancreatic juice from PM pigs were higher ($P < 0.01$) than for CM pigs (Fig. 5-3). In contrast, the specific and total activities of carboxyl ester hydrolase in pancreatic juice from CM pigs were much higher ($P < 0.001$) than for PM pigs. The specific activity of lipase was higher ($P < 0.001$) in pancreatic juice

from PM than from CM pigs. However, the total activities of lipase did not differ ($P > 0.05$) between CM and PM pigs. The specific and total activities of colipase in pancreatic juice from CM pigs were greater ($P < 0.01$) than in pancreatic juice from PM pigs.

A considerable amount of trypsin and chymotrypsin activity was present in nonactivated pancreatic juice from PM pigs (Fig. 5-3); trypsin activity was not detected in nonactivated pancreatic juice from CM pigs. Less than 1 U L^{-1} of chymotrypsin activity was detected in nonactivated pancreatic juice from CM pigs.

The relationships and correlation coefficients between the concentration of protein in pancreatic juice and the specific activities of trypsin and the relationship between the specific activities of one enzyme with the other enzymes are shown in scatter plots in Fig. 5-4. These relationships are presented to illustrate differences within each method and to demonstrate that method can affect the components of pancreatic juice and therefore may affect interpretation of results. In PM pigs, specific trypsin activity ranged from 513 to 1922 U L^{-1} and its activity was weakly related ($P < 0.05$) to the concentration of protein. However, specific activity of trypsin ranged from 1125 to 11221 U L^{-1} in CM pigs and was directly related ($P < 0.001$) to protein concentration. For PM pigs, the correlation coefficient between specific trypsin and chymotrypsin activity was 0.36 ($P > 0.05$) and in CM pigs there was no relationship. A plot of specific lipase activity in relation to colipase activity for PM pigs indicated that there was a weak ($P > 0.05$) relationship between lipase and colipase activity. Colipase activities in pancreatic juice from PM pigs were usually low. In contrast, a linear relationship ($P < 0.001$) was observed between lipase and colipase activity in CM pigs.

D. Discussion

The differences in the volume of pancreatic juice secreted by pigs prepared with the Pouch or Catheter Method (Fig. 5-1) may be a result of the surgical preparation of the pigs. When a catheter is placed in the pancreatic duct, the duct is ligated just before entry into the duodenum and the duodenal papilla is bypassed. Removal of the function of the papilla may increase secretion, however, this aspect remains to be investigated and has not been discussed in the literature. The effect of placing a catheter in the pancreatic duct on secretin secretion, which is primarily resposible for stimulating fluid and bicarbonate secretion, should be investigated (Brannon 1990; Pierzynowski et al. 1995).

In the construction of the isolated duodenal pouch, most of the extrinsic nerves leading from the stomach to the pancreas via the duodenum must be cut; regulatory mechanisms may be affected and pancreatic secretion altered (Thomas 1959; Pierzynowski et al. 1988). In addition, the construction of a duodenal pouch removes a segment of duodenum from exposure to acidic digesta leaving the stomach. Therefore, the secretion of secretin from endocrine cells in the pouch may not occur or may be lower. Overall secretion of secretin may be reduced and this may lead to a decline in the volume of pancreatic juice secreted (Ushijima et al. 1984; Krzemiński et al. 1990). This may have, in part, been responsible for the lower volume of pancreatic juice in the PM pigs (Fig. 5-1 and 5-2).

The highly variable and irregular pattern of pancreatic secretion and variation in enzyme activities in this study (Fig. 5-1, 5-2 and 5-3) could have been due to a number of factors. These include parasympathetic stimulation, behaviour (posture, movement and sleep) and others. However, behavioral events were not recorded in this study but they may

explain some of the variation in pancreatic secretion and should be monitored in future studies. The secretion rates of pancreatic juice and enzyme activities were highly variable in other studies in which the Pouch Method (Zebrowska and Low 1987) and the Catheter Method (Partridge et al. 1982) were used. In this study, most variability in the volume of pancreatic juice secreted was observed in the CM pigs (Fig. 5-1). There was a large amount of variation between pigs for each hourly collection. The hourly rates of pancreatic juice secreted by CM pigs in this study were within the range of values reported for pigs of similar BW and prepared with the Catheter Method (Partridge et al. 1982). The diurnal pattern of secretion in each experimental period for each pig was usually different over the whole collection period (Fig. 5-2).

The substantially lower ($P < 0.01$) pH of pancreatic juice secreted by PM pigs (Fig. 5-1) may have been due to the presence of hydrolysis products, likely peptides and amino acids, released from proteolysis of pancreatic enzymes by active proteases. A lower level of plasma secretin and consequently bicarbonate concentration which was lower ($P > 0.05$) in pancreatic juice from PM pigs (Fig. 5-3) may have also been partly responsible.

The realization that active proteases were present in pancreatic juice from dogs prepared for total collection of pancreatic juice with the Pouch Method came in early experiments on pancreatic secretion (Dragstedt et al. 1930; Scott 1940). It has also been mentioned and discussed several times in the literature during the last few decades (Woods and Foster 1963; Ternouth and Buttle 1973; Makkink et al. 1990; St-Jean et al. 1992). In several studies in which the Pouch Method was used to collect pancreatic juice from pigs and ruminants, researchers did not carry out an activation step (Zebrowska et al. 1983;

Zebrowska and Low 1987; Walker and Harmon 1995). Makkink et al. (1990) reported that trypsin and chymotrypsin were fully active in pancreatic juice from a PM pig (a one-way valve was not used), therefore activation was unecessary. The authors did not state whether or not there was any evidence of backflow of digesta into the duodenal pouch. In Chapter 2, an activation step was carried out but we did not, based on the results of Hee (1984), anticipate that there would be any substantial protease activity in pancreatic juice from the duodenal pouch.

Hee (1984) reported that flushing the duodenal pouch with saline (the frequency was not discussed) to remove free enterokinase prevented activation of trypsinogen. Further investigation on whether or not special precautions or protease inhibitors need to be used during collection of pancreatic juice from animals prepared with the Pouch Method is required. The use of protease inhibitors may have implications for the determination of trypsin and chymotrypsin activities.

Trypsinogen and chymotrypsinogen in pancreatic juice from PM pigs had been fully activated to trypsin and chymotrypsin (Fig. 5-3). Similar results were reported by Ternouth and Buttle (1973), addition of enterokinase did not increase proteolytic enzyme activity in pancreatic juice from PM calves. When the activation step was carried out, specific and total chymotrypsin activities were reduced (Fig. 5-3, $P < 0.05$). These reductions were likely due to proteolysis or inactivation by active proteolytic enzymes secreted in the duodenal pouch.

The similar specific chymotrypsin activities in pancreatic juice from CM and PM pigs and the higher ($P < 0.05$) specific trypsin activity in pancreatic juice from CM than

from PM pigs suggests that chymotrypsin is more resistant to proteolysis or inactivation than trypsin (Goldberg et al. 1969; Low 1982). Conflicting results were reported by Khayat and Christophe (1969); more trypsin than chymotrypsin activity was maintained in intestinal washings incubated at 37°C. Some of the difference between the total activities of trypsin and chymotrypsin may have been due to hormonal effects; perhaps there was an effect of the collection method on cholecystokinin level. Cholecystokinin is responsible for mediating the secretion of pancreatic proteases (Brannon 1990). Although this is speculative it should be investigated further and it may explain some of the differences observed between the methods.

The higher ($P < 0.001$) specific amylase and lipase activities in pancreatic juice from PM than from CM pigs (Fig. 5-3) suggest that amylase and lipase may be more resistant to inactivation or proteolysis than other enzymes in pancreatic juice. Amylase contains divalent cations, such as calcium, which confer resistance to proteolysis (Stein and Fischer 1958). In support of increased resistance, 74% of amylase activity secreted into the duodenum survived transit to the distal ileum in intubation studies with humans (Layer et al. 1986). However, 99% of lipase activity was inactivated during passage from the duodenum to the ileum (Layer et al. 1986). Whether or not the same situation occurs in the pig remains to be determined. In contrast, in vitro experiments with activated pancreatic juice demonstrated that lipase was relatively resistant to inactivation until pancreatic proteases had been active for some time (Borgström et al. 1993). However, lipase is less resistant to inactivation than either trypsin or chymotrypsin (Pelot and Grossman 1962).

Carboxyl ester hydrolase and colipase were likely inactivated or digested (Fig. 5-3) to a large extent by active proteolytic enzymes in the duodenal pouch of PM pigs. Trypsin has been shown to be primarily responsible for its autoactivation and inactivation of itself, chymotryspin, amylase and lipase (Khayat and Christophe 1969). The relationship between lipase and colipase activities (Fig. 5-4) clearly illustrates that colipase was inactivated; colipase activity usually did not increase as lipase activity increased in pancreatic juice from PM pigs. Conversely, in agreement with the results of Pierzynowski et al. (1995), there was a direct relationship between lipase and colipase activities in CM pigs. Procolipase is rapidly activated by trypsin to colipase before trypsin and chymotrypsin activities plateau. Colipase is inactivated to a large extent during and following activation of these two proteolytic enzymes (Borgström et al. 1993).

Despite the relatively large loss of enzymatic activity, i.e., proteolysis or inactivation, pancreatic juice from PM pigs contained more protein than pancreatic juice from CM pigs (Fig. 5-3 and 5-4). This was likely due to the presence of sloughed epithelial cells and mucin secreted into the pouch (Ternouth and Buttle 1973; Zebrowska et al. 1983; Hee 1984). Pancreatic juice from PM pigs was more viscous and contained more cell debris than pancreatic juice from CM pigs. Similar results were also reported by Hee (1984).

The two commonly used methods used to prepare pigs for total collection of pancreatic juice have strengths and weaknesses. The Pouch Method can be used in long term experiments (months) and morbidity and mortality may be lower than when the Catheter Method is used. However, preparing the duodenal pouch is a relatively severe procedure and may interfere with the regulatory and feedback mechanisms of pancreatic

secretions. Furthermore, there may be an effect on gastrointestinal hormone levels especially secretin. In addition, based on observations made in this study, a longer post-surgical recovery time is required when the Pouch Method is used. The physiological consequences of forming the duodenal pouch and the problem of proteolytic enzyme activation and consequent effects on the activities of other enzymes in pancreatic juice from the duodenal pouch remain to be investigated further. Based on our experience, pigs with a catheter in the pancreatic duct require very intensive management and this technique is very well suited for short term studies similar to this experiment. However, the effect on pancreatic secretions and on gastrointestinal hormones of the presence of a catheter in the pancreatic duct and bypassing the duodenal papilla remains to be investigated.

In conclusion, there are large and significant differences in the volume of pancreatic juice secreted, pH of pancreatic juice and specific and total activities of several enzymes when the Pouch and Catheter Methods are used. These differences may affect the response of the exocrine pancreas to changes in diet composition, feeding regimen or application of a particular hormone, agonist or inhibitor. The Catheter Method appears to be a more suitable method than the Pouch Method to collect pancreatic juice in growing pigs. The catheter method is less invasive and most of the neuronal and hormonal mechanisms controlling exocrine pancreatic secretions likely remain intact. The confounding effects of active proteolytic enzymes are avoided when this method is used.

E. Implications

The Catheter Method may be the prefered method to collect pancreatic juice from growing pigs, this method can be successfully used in short term studies provided the pigs are well cared for and managed. The Pouch Method needs to be investigated further and steps need to be taken to minimize the effect of active proteolytic enzymes in pancreatic juice from pigs prepared with this method. If only the secretions of amylase, lipase and chymotrypsin are investigated, this method may be representative because these enzymes appear to not be affected by the presence of active proteolytic enzymes. Further studies are required to investigate the adaptation of exocrine pancreatic secretion to different conditions when either method is used.

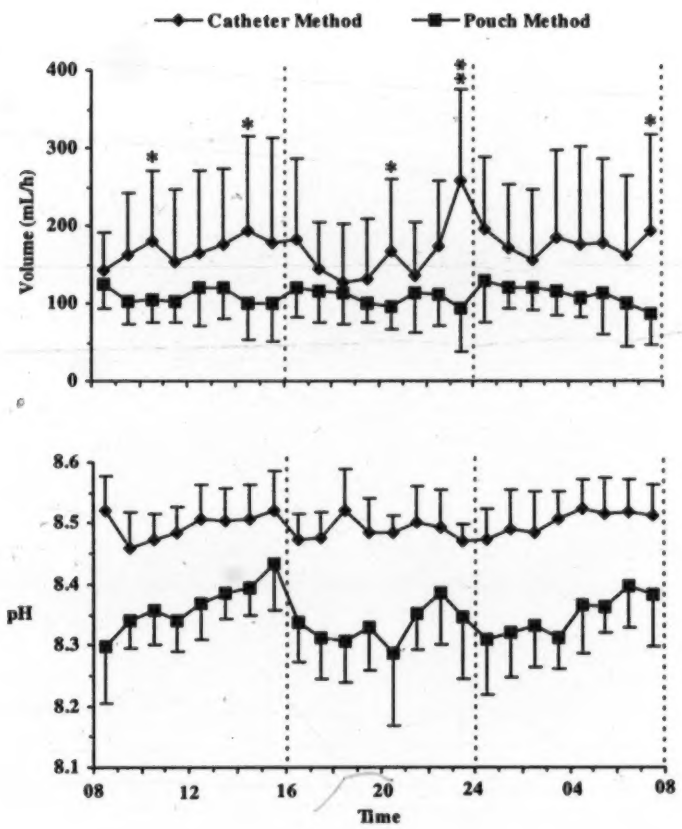

Fig. 5-1. Diurnal pattern of secretion of pancreatic juice and pH of pancreatic juice from pigs prepared with the Pouch or Catheter Method. Secretion rates differ between methods: $*P < 0.05$, $**P < 0.01$. For pH, the methods differ ($P < 0.01$) at each hour of collection. Each point is the mean of nine observations and the standard deviations. The dashed vertical lines indicate feeding times.

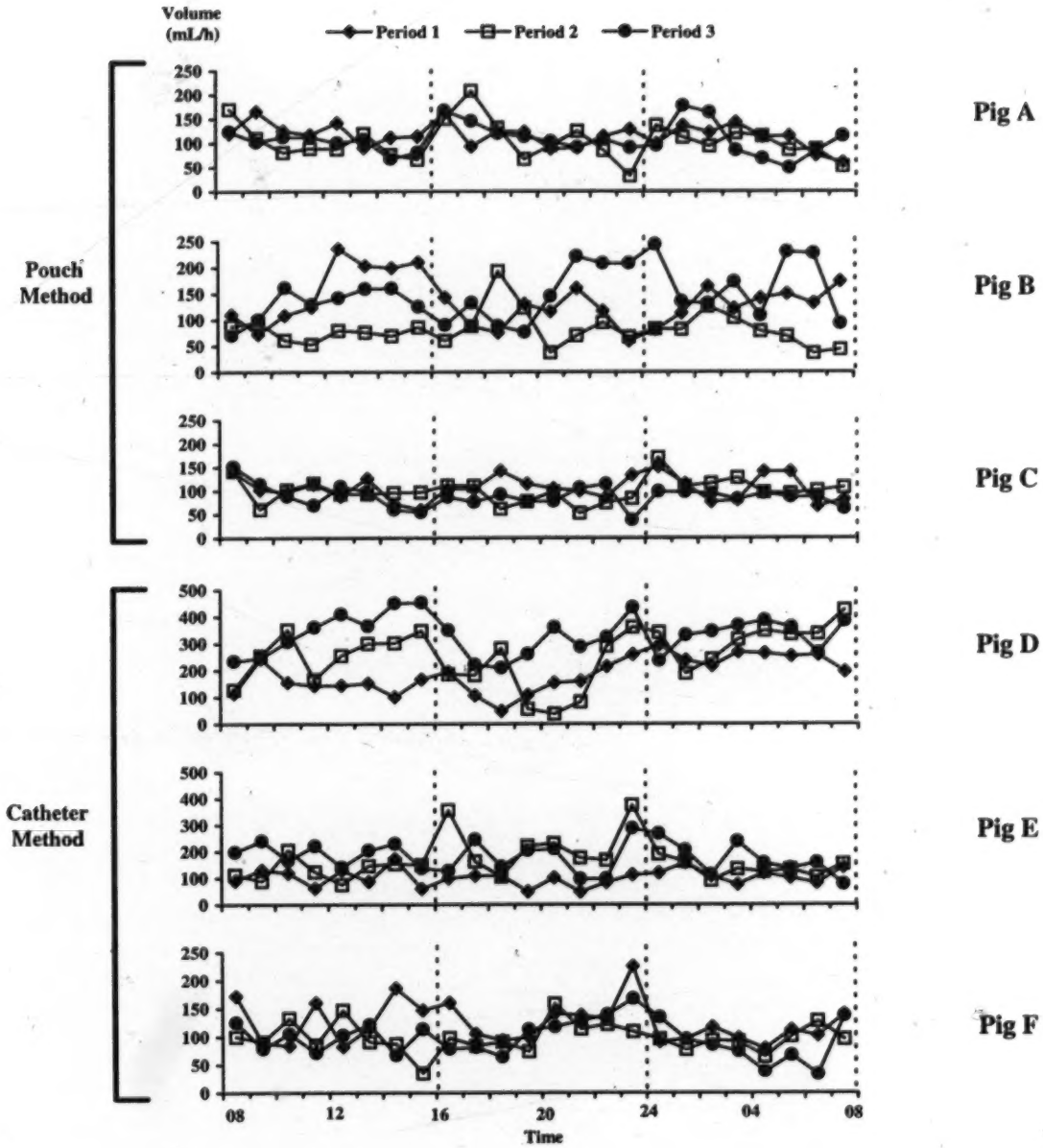

Fig. 5-2. Diurnal pattern of the secretion of pancreatic juice in Periods 1, 2 and 3 in pigs prepared with the Pouch (Pigs A, B and C) or Catheter Method (Pigs D, E and F). The dashed vertical lines indicate feeding times.

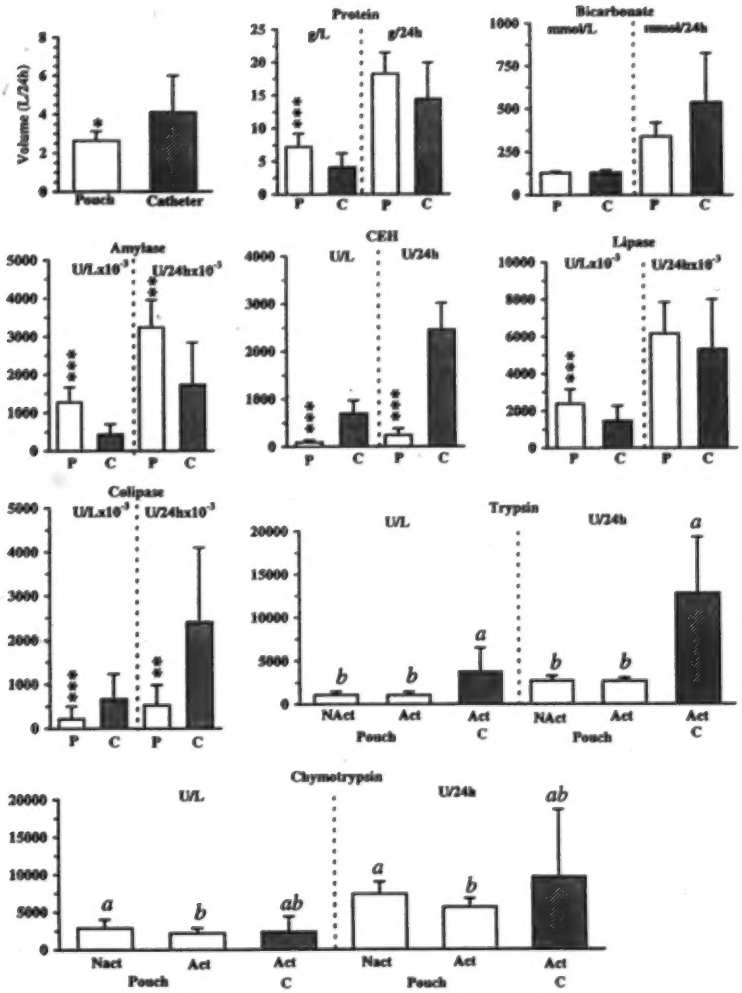

Fig. 5-3. Daily volume of pancreatic juice secretion, concentrations and daily outputs of protein and bicarbonate and specific and total activities of amylase, carboxyl ester hydrolase (CEH), lipase and colipase in pancreatic juice from pigs prepared with the Pouch (P) or Catheter (C) Method. Trypsin and chymotrypsin activities in nonactivated (Nact) and activated (Act) pancreatic juice from pigs prepared with the Pouch Method are presented. Virtually no activities were detected in nonactivated pancreatic juice from Catheter Method pigs (See Results). Each point is the mean of nine observations and the standard deviation. Means differ between methods: * or a versus b $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Comparisons were only made between concentration, output and specific activities or total activities (indicated by the dashed line).

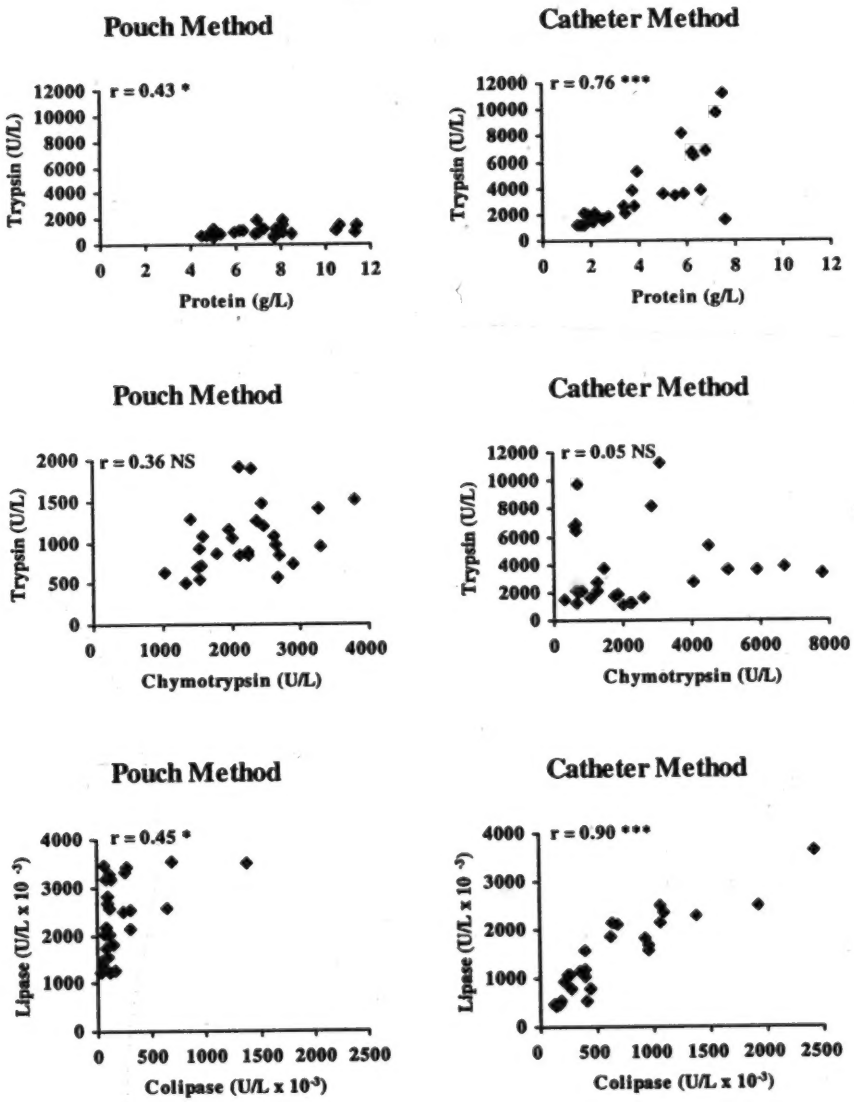

Fig. 5-4. Scatter plots (n = 27) and correlation coefficients between the specific activities of trypsin and the concentration of protein, the specific activities of trypsin and chymotrypsin as well as the specific activities of lipase and colipase in pancreatic juice from pigs prepared with either the Pouch or the Catheter Method. Correlation coefficients are significant: $*P < 0.05$, $***P < 0.001$.

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CHAPTER 6

ELECTROPHORETIC SEPARATION OF PROTEOLYTIC ENZYMES IN PANCREATIC JUICE COLLECTED WITH THE POUCH OR CATHETER METHOD

A. Introduction

Two commonly used methods are used to collect pancreatic juice in pigs. One method, referred to as the Catheter Method (CM), involves surgical placement of a catheter into the pancreatic duct and insertion of a small T-cannula into the duodenum to return pancreatic juice. The second method, referred to as the Pouch Method (PM) also involves permanent re-entrant diversion of pancreatic juice. However, pancreatic juice is collected from a duodenal pouch into which the pancreatic duct enters. These methods were discussed and compared in Chapter 5. A major difference between these methods was that trypsin and chymotrypsin were fully active in nonactivated pancreatic juice from PM pigs whereas virtually no trypsin or chymotrypsin activity was detected in pancreatic juice from CM pigs before activation with enterokinase (Chapter 5).

Recently, a relatively simple technique which facilitates the separation and identification of porcine pancreatic enzymes and their isozymes was developed. This procedure involves the separation of pancreatic enzymes by electrophoresis followed by the identification of the location of the enzymes in the gel using a specific substrate that

produces a color reaction (Ohlsson et al. 1987). This method has been successfully used to study secretion of pancreatic enzymes in pigs (Weström et al. 1987; Pierzynowski et al. 1993).

The objective of this study was to further investigate the enzyme activities in pancreatic juice from PM and CM pigs. This was carried out using separation by electrophoresis and N-acetyl-DL-phenylalanine-b-naphthyl ester (Ac-Phe-bNE) as a substrate to identify the isozymes of chymotrypsin, trypsin and only one elastase.

B. Materials and Methods

Collection of Pancreatic Juice

The surgical preparation of the pigs and sampling procedures are described in detail in Chapters 4 and 5. The samples were frozen at -80°C and stored for 6 months until analyses. Samples of pancreatic juice from the first 8-h sampling period of the first experimental period from all six pigs were used for analyses.

Electrophoresis and Enzyme Activities

Both nonactivated and enterokinase activated samples of pancreatic juice were used in the separation by electrophoresis and enzyme assays. Proteolytic enzymes in pancreatic juice were activated according to procedures modified from Glazer and Steer (1977). Five microliters of a solution with enterokinase [1 mg porcine enteropeptidase (code E0632, Sigma Chemical, St. Louis, MO) mL^{-1} 154 mM sodium chloride] was added to 50 µL undiluted pancreatic juice and incubated at 37°C for 30 min. Separation by electrophoresis was carried out on 10 µL of nonactivated and activated pancreatic juice. The separation was

performed in 1% agarose gel (HSB, Litex, Glostrup, Denmark) in 37 mM calcium-veronal buffer, pH 8.6, on a plastic support (Gel-Bond, Marine Colloids, Rockland, ME) at 20 V cm^{-1} as described by Ohlsson et al. (1987). The separations were performed in triplicate under the same conditions.

After electrophoresis, proteolytic enzyme activities were localized directly on the agarose gel. Twelve milligrams of Ac-Phe-bNE (code A7512, Sigma Chemical) in 5 mL dimethyl formamide and 50 mg o-dianisidine (Fast Blue B, code D3252, Sigma Chemical) were diluted to 50 mL in 200 mM Tris-hydrochloride buffer (pH 7.8). The solution was added to the gel and incubated at room temperature (22°C) for 1 h (Ohlsson et al. 1987). The enzyme bands were identified according to their mobility (in order from the anode to the cathode): chymotrypsin C, anodal trypsin, chymotrypsin B, chymotrypsin A, elastase II and cathodal trypsin (Ohlsson et al. 1987; Weström et al. 1987; Pierzynowski et al. 1993). The two isozymes of trypsin are discriminated from each other by their movement during electrophoretic separation rather than as trypsin A or B (Voytek and Gjessing 1971).

C. Results

Representative photographs of the gels derived from both nonactivated and enterokinase activated pancreatic juice from three pigs prepared with the Pouch and Catheter Methods are shown in Fig. 6-1. Based on the intensity of the color reaction, this qualitative enzyme assay indicated that a considerable amount of chymotrypsin C, anodal trypsin, chymotrypsin B, chymotrypsin A, elastase II and cathodal trypsin activities were present in nonactivated pancreatic juice from PM pigs. In contrast, only trace amounts of

chymotrypsin A and elastase II were identified in pancreatic juice from CM pigs. After enterokinase activation, the amounts of chymotrypsin C and cathodal trypsin appeared to be lower in nonactivated pancreatic juice from PM pigs, while the activities of anodal trypsin, chymotrypsin B, chymotrypsin A and elastase II appeared to increase. As expected, enterokinase activation of pancreatic juice from CM pigs resulted in the detection of high amounts of all the proteolytic enzymes. The amounts of anodal trypsin, chymotrypsin A and chymotrypsin B and elastase II appeared to be higher in activated pancreatic juice from CM than from PM pigs.

D. Discussion

The large amount of proteolytic enzymes observed in nonactivated pancreatic juice from PM pigs (Fig. 6-1) is in agreement with results presented in Chapter 5. It appears that the activation cascade, presumably started by the activation of trypsinogen by enterokinase in the duodenal pouch, was largely completed during the collection period. Similar results were reported by Ternouth and Buttle (1973).

The total amount of chymotrypsin in nonactivated pancreatic juice from PM pigs (2880 U L^{-1}) declined ($P < 0.05$) after enterokinase activation (2200 U L^{-1}, Chapter 5). This decline appears to be due to a reduction in the amount of chymotrypsin C (Fig. 6-1). There was a small reduction in the amount of trypsin when pancreatic juice from PM pigs was activated (Chapter 5), which appears to be due to a reduction in the amount of cathodal trypsin.

Less than 1 U L^{-1} of chymotrypsin activity was measured in nonactivated pancreatic juice from CM pigs (Chapter 5). The presence of this small amount of activity can be attributed to chymotrypsin A based on electrophoretic localization in this study. This small amount of chymotrypsin A as well as of elastase II may have been the result of handling (e.g., bacterial contamination). Some autoactivation or proenzyme activity against the low molecular weight substrate used (Ac-Phe-bNE) may also have occurred.

In Chapter 5, in which enzyme activities were determined in pancreatic juice from both groups of pigs, trypsin activity in pancreatic juice from CM pigs was higher ($P < 0.05$, 3682 U L^{-1}) than in both activated and nonactivated pancreatic juice from PM pigs (1031 and 1053 U L^{-1}, respectively). These observations are confirmed by the results of this study (Fig. 6-1). Trypsin has been shown to be primarily responsible for the inactivation of pancreatic enzymes in the small intestine (Khayat and Christophe 1969) and was likely responsible, in part, for the lower trypsin activity in pancreatic juice from the duodenal pouch.

The results of this study support the premise that if the Pouch Method is used to collect pancreatic juice, steps need to be taken to prevent the activation of proteolytic enzymes. Hee (1984) reported that flushing the duodenal pouch with saline (the frequency was not discussed), to remove free enterokinase, prevented the activation of trypsinogen. Hee (1984) also concluded that flushing the pouch with Trasylol (aprotinin, a trypsin inhibitor) was unnecessary because trypsin activity was not detected in secretions collected with or without the use of Trasylol. Pancreatic juice used in this study was obtained from pigs in which the duodenal pouch cannula was flushed out with saline every second day to

ensure the pouch was free of cell debris and mucin and to keep the one-way valve clean (Chapter 5). Further studies are required to investigate the reactions taking place in the duodenal pouch and factors that influence these such as the length of time pancreatic juice spends in the pouch.

In conclusion, the results of this study demonstrated that proteolytic enzymes in pancreatic juice from pigs prepared with the Pouch Method were nearly fully active or were fully active. When activation with enterokinase was carried out, further inactivation and(or) breakdown occurred for chymotrypsin C and cathodal trypsin. In addition, some inactivation and(or) breakdown of proteolytic enzymes in pancreatic juice occurred during collection of pancreatic juice from PM pigs.

E. Implications

The results of this study confirm that activation of trypsinogen to trypsin with enterokinase and chymotrypsinogen to chymotrypsin with trypsin are not necessary in pancreatic juice from pigs prepared with the Pouch Method. The Catheter Method may be the preferred method to collect pancreatic juice from pigs, the confounding effects of active proteolytic enzymes is avoided.

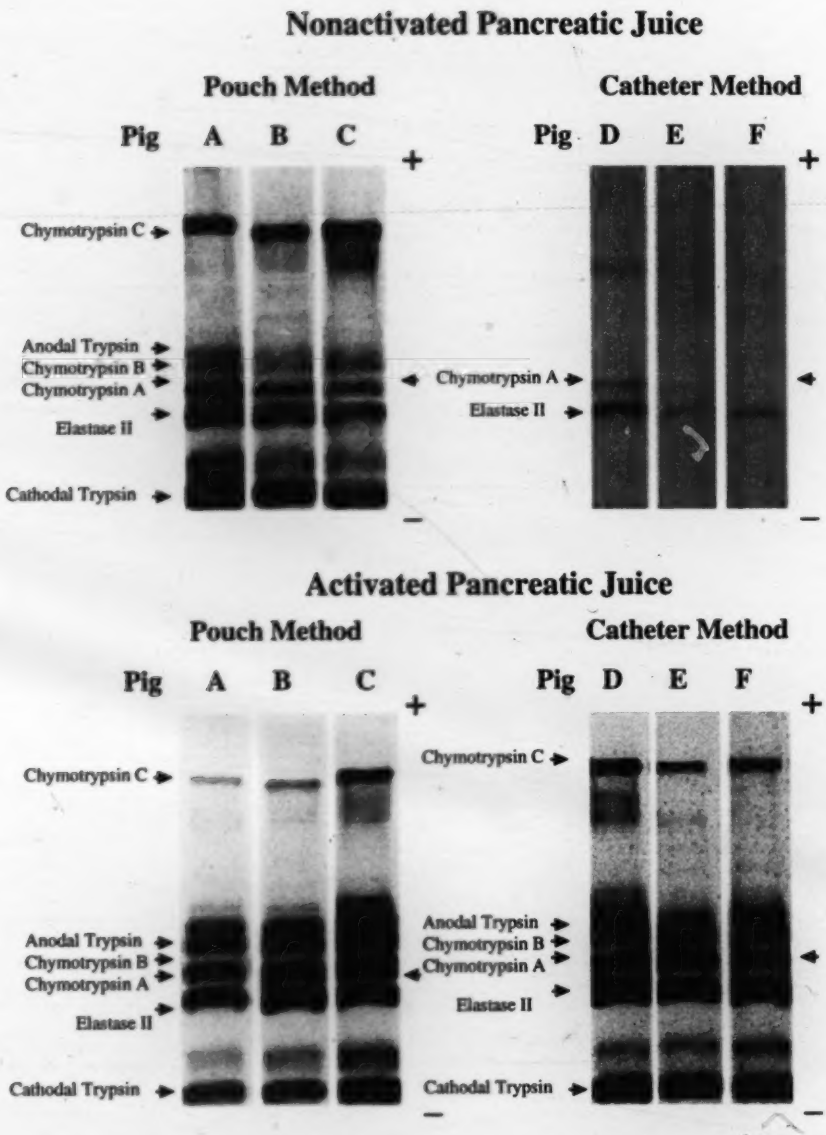

Fig. 6-1. Patterns identifying the isozymes of pancreatic proteolytic enzymes, following separation by electrophoresis and hydrolysis of the substrate Ac-Phe-bNE, in nonactivated and enterokinase activated pancreatic juice from growing pigs prepared with the Pouch (Pigs A, B and C) or Catheter Method (Pigs D, E and F). Site of sample application is indicated by an arrowhead on the right side. The anode (+) is at the top of the figure.

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CHAPTER 7

PANCREATIC SECRETION OF ZINC AND CARBOXYPEPTIDASE A AND B IN GROWING PIGS

A. Introduction

Pancreatic juice is a complex solution which contains various enzymes and ions. Several studies have demonstrated that the concentration of zinc in pancreatic juice is higher than in other gastrointestinal secretions (Sullivan et al. 1974; Vallee and Falchuk 1993). Zinc is found complexed to proteins rather than as a free ion in solution and it has a catalytic, structural or catalytic and structural role in over 300 metalloenzymes representing 50 different types of enzymes (Vallee and Falchuk 1993). These include carboxypeptidase A and B which are exopeptidases in pancreatic juice that catalyze hydrolysis of C-terminal amino acids in polypeptide chains and differ in their specificities. Zinc has a catalytic role in carboxypeptidase A and B (Vallee and Falchuk 1993). The concentration of zinc and the activities of carboxypeptidase A and B are highly correlated in pancreatic juice from rats (Berger and Schneeman 1986). However, the correlation between carboxypeptidase A and B activities and the concentration of zinc in pancreatic juice has not been determined in the pig and may be of physiological significance. In addition, the diurnal pattern of zinc secretion and carboxypeptidase A and B have not been determined.

The objective of the present study was to determine the diurnal pattern of exocrine pancreatic secretion of zinc and the zinc-dependent metalloenzymes, carboxypeptidase A and B, in growing pigs fed a grower-finisher diet supplemented with zinc to exceed Danish standards for growing pigs (Andersen and Just 1983). Furthermore, these studies enabled us to compare the daily pancreatic secretion of zinc to the daily intake of zinc.

B. Materials and Methods

Animals and Diets

A detailed description of the pigs, diets, and collection and handling of pancreatic juice is presented in Chapter 4. Zinc, in the form of zinc oxide, supplied by the premix was 80 mg kg^{-1} diet and the zinc content of the diets was 110 mg kg^{-1}. The pigs were fed 1.65 kg d^{-1} in three meals, equal amounts, at 0800, 1600 and 2400 h. The daily intake of zinc was 182 mg.

Chemical Analyses and Enzyme Activities

Prior to analyses, the hourly pancreatic juice samples were thawed in warm water (approximately 30°C) and immediately placed in a water bath with ice. The samples were proportionally pooled over 2 h to give a sample size of 6 mL. From the pooled 2-h sample, two 1 mL samples were pipetted into vials for the determination of protein and carboxypeptidase A and B activities. Therefore, 4 mL of pancreatic juice were wet ashed prior to determination of zinc.

The concentration of protein in pancreatic juice was measured as described in Chapter 4.

Zinc content was analysed by atomic absorption spectrometry (PU 9400 X, Philips Scientific, Cambridge, UK). Samples were wet ashed by addition of concentrated nitric acid (14 M, 1:1 vol/vol) and exposed to increasing temperatures. Measurements were calibrated on Tritisol (a prepared standard, Merck, Darmstadt, Germany); standard curves were compared to independently produced zinc chloride solutions before each analysis.

Carboxypeptidase A and B differ in their specificities and will not catalyze the hydrolysis of C-terminal amino acids if certain amino acids are present. For carboxypeptidase A the C-terminal residue (R_n) cannot be arginine, lysine or proline and the next amino acid (R_{n-1}) also cannot be proline; carboxypeptidase B cleaves if R_n is arginine or lysine and R_{n-1} cannot be proline (p. 113, Voet and Voet 1990).

Procarboxypeptidase A was activated to carboxypeptidase A with trypsin. Pancreatic juice was diluted 10 times in a buffer [5 mM tris(hydroxymethyl)aminomethane (code 8382, Merck, Darmstadt, Germany), 45 mM sodium chloride and pH 7.5]. Subsequently, 50 µL of a trypsin solution [50 mg bovine trypsin (code T8253, Sigma Chemical, St. Louis, MO) dissolved in 50 mL tris buffer] was added to 1 mL of diluted pancreatic juice and the solution incubated at 37°C for 15 min. Carboxypeptidase A activity was measured according to procedures described by Yamasaki et al. (1963) with minor modifications. Activated pancreatic juice (100 µL) was added to 5 mL of a substrate solution containing 5 mM hippuryl-DL-phenyllactic

acid (code H9755, Sigma Chemical). Using a Titralab pH-stat (Radiometer, Copenhagen, Denmark) the hydrolysis was followed by continuous titration with 0.1 M sodium hydroxide at 25°C .

The inactive procarboxypeptidase B in pancreatic juice was activated to carboxypeptidase B with trypsin. Pancreatic juice was diluted 5 to 20 times in a buffer [27.5 mM tris(hydroxymethyl)aminomethane (code 8382, Merck, Darmstadt, Germany), 110 mM sodium chloride and pH 7.65]. Thereafter, 50 µL of a trypsin solution [50 mg trypsin (code T8253, Sigma Chemical) dissolved in 50 mL buffer] was added to 1 mL of diluted pancreatic juice and the solution incubated for 20 min at 25°C. The method of Appel (1974) was used to measure carboxypeptidase B activity.

Enzyme activity in pancreatic juice was expressed as units (U) per liter (specific activity) and per 2 or 24 h (total activities). One U of enzyme activity was defined as the hydrolysis of 1 µmol of substrate in 1 min. Total enzyme activities were calculated as specific activity x volume of pancreatic juice secreted per 2 or 24 h.

Statistical Analyses

Analysis of variance was carried out according to procedures described in Chapter 5. The 2-h secretion rates, concentration and secretion of protein and zinc and specific and total activities of carboxypeptidase A and B measured in PM and CM pigs were subjected to ANOVA for each 2-h collection interval of the 24-h collection.

C. Results

Within each collection method (Pouch or Catheter) there was no effect ($P > 0.05$) of diet or experimental period on volume of pancreatic juice secreted, protein and zinc concentrations and secretion and specific or total activities of carboxypeptidase A and B.

The diurnal patterns of the 2-h secretion of pancreatic juice, protein and zinc are shown in Fig. 7-1. The volume of pancreatic juice secreted by CM pigs was sometimes higher ($P < 0.05$) and more variable than the volume of pancreatic juice secreted by PM pigs. The diurnal patterns of the secretion of protein and zinc in pancreatic juice from both groups of pigs peaked within 2 h after feeding. Protein and zinc secretions were usually higher ($P < 0.05$) in pancreatic juice from PM than from CM pigs.

The patterns of secretion of total carboxypeptidase A and B activities are presented in Fig. 7-2. In both PM and CM pigs, carboxypeptidase A activity was highest within 2 h after feeding and was usually higher, but only significant between 1600 and 1800 h, in pancreatic juice from PM than from CM pigs. Carboxypeptidase B secretion also reached its highest levels for PM and CM pigs within 2 h of feeding and was often substantially higher ($P < 0.05$) in pancreatic juice from PM than from CM pigs. The diurnal patterns of total carboxypeptidase A and B activities were similar to those of protein and zinc (Fig. 7-1 and 7-2).

The volume of pancreatic juice and concentrations and flows of zinc and protein and specific and total carboxypeptidase A and B activities in pancreatic juice from pigs prepared with the Pouch or the Catheter Method are shown in Table 7-1. The daily

volume of pancreatic juice from CM pigs was higher ($P < 0.05$), 4.1 versus 2.6 L 24 h^{-1}, respectively, than PM pigs. The concentrations and daily secretions of protein were greater ($P < 0.01$) in pancreatic juice from PM pigs. The average concentration of zinc (1.00 and 0.41 mg L^{-1}, respectively) and daily secretion of zinc (2.54 and 1.50 mg 24 h^{-1}, respectively) in pancreatic juice were also higher ($P < 0.0001$) in PM than in CM pigs. In the present experiment, the percentage of daily zinc intake secreted in pancreatic juice was calculated to be 1.40 and 0.82% for PM and CM pigs, respectively.

The specific activities of carboxypeptidase A were greater ($P < 0.0001$) in pancreatic juice from PM than from CM pigs (Table 7-1). However, total activities of carboxypeptidase A did not differ between both groups of pigs. The specific and total activities of carboxypeptidase B in pancreatic juice from PM pigs were higher ($P < 0.01$) than in pancreatic juice from CM pigs.

The correlation coefficients between zinc, protein and specific and total carboxypeptidase A and B activities are presented in Table 7-2. For PM pigs, the highest correlation coefficients were observed between protein and zinc concentration and secretion and protein and zinc concentration and secretion versus specific and total carboxypeptidase A activities. The highest correlation coefficients for CM pigs were observed between zinc concentration and specific carboxypeptidase A activity, protein concentration and zinc concentration and protein concentration and specific carboxypeptidase B activity.

D. Discussion

The differences observed between the two groups of pigs in the volume of pancreatic juice secreted and protein concentration and secretion (Fig. 7-1 and Table 7-1) were discussed in Chapter 5.

The concentrations of zinc in pancreatic juice from CM pigs in this study (Table 7-1) are within the range of those determined in an experiment with 5-kg pigs which were anaesthetized and pancreatic secretions were stimulated with an injection of secretin (Sullivan et al. 1981). However, these studies are difficult to compare given the different BW of the pigs and methodology used. In a recent study in which growing pigs were prepared with the Pouch Method, total daily secretion of zinc in pancreatic juice was 4.0 mg and total dietary intake of zinc 60 mg (Chavez et al. 1996). A similar daily rate of zinc excretion was observed in this experiment with pigs prepared with the Pouch Method (Table 7-1) even though zinc intake was twice as high (182 mg d^{-1}).

In the present study, the pattern of zinc secretion in pancreatic juice from growing pigs was parallel to the secretion of protein and the zinc-dependent enzymes carboxypeptidase A and B (Fig. 7-1 and 7-2). Similar results were reported by Berger and Schneeman (1986). Following intravenous injection of ^{65}Zn in growing pigs with a catheter in the pancreatic duct, Pekas (1966) observed a very close relationship between hourly concentration of protein, up to 7 h following injection, and ^{65}Zn counts suggesting that zinc was bound to proteins in pancreatic juice. Furthermore, approximately 95% of the ^{65}Zn activity in pancreatic juice was precipitated by acetone and assumed to be protein bound (Pekas 1966).

Increased pancreatic secretions of digestive enzymes following feeding in pigs has been observed in several studies (e.g., Hee et al. 1988; Thaela et al. 1995) and was also observed in this study (Fig. 7-2). This is the first study in which the diurnal patterns of zinc and carboxypeptidase A and B secretion have been investigated. The results of study demonstrate that the secretion rates change throughout the day and are quite variable in pigs (Fig. 7-1 and 7-2).

High correlation coefficients between zinc concentration, protein concentration and carboxypeptidase A and B activities have been observed in previous experiments with rats (Berger and Schneeman 1986). However, the correlation coefficients were lower in this study (Table 7-2). The rat experiments were of short duration, 2 h of collection, and the experimental conditions were standardized. These factors may have been responsible for some of the differences between the correlation coefficients. Reinstein et al. (1987) concluded that zinc in pancreatobiliary fluid is associated primarily with carboxypeptidase A and B.

Preliminary analyses indicated that carboxypeptidase A and B were fully active in nonactivated pancreatic juice from PM pigs (data not shown). In addition, the activation step with trypsin did not result in increased activity (data not shown). These results are in agreement with our previous observations; proteolytic enzymes in pancreatic juice from PM pigs are fully active (Chapter 5). The elevated secretion of carboxypeptidases A and B may be explained, in part, by the higher concentration of protein in pancreatic juice from PM pigs (Fig. 7-1 and Table 7-1).

The difference in zinc secretion in pancreatic juice between PM and CM pigs (Fig. 7-1 and Table 7-1) was likely related to physiological changes induced by the two collection methods as discussed in Chapter 5. The higher concentration of zinc in pancreatic juice from PM pigs was likely due to the presence of sloughed epithelial cells. No attempt was made to filter or purify the samples of pancreatic juice from PM pigs but it would be useful to quantify the amount of zinc associated with sloughed epithelial cells originating from the duodenal pouch.

In this experiment, zinc intake was adequate and therefore the rate of synthesis and level of metallothionein, a protein which is rich in cysteine and binds a large amount of zinc (Cousins 1985; Vallee and Falchuk 1993), was likely high. Therefore mucosal cells contained a large amount of zinc which was released into the duodenal pouch. In addition, cysteine-rich intestinal protein, which binds zinc during transmucosal transport, may also contribute zinc to the duodenal pouch when epithelial cells are sloughed off (Hempe and Cousins 1991).

The contribution of pancreatic zinc secretion to the maintenance of zinc homeostasis may be relatively low. The concentration of zinc in pancreaticobiliary fluid from rats was slightly elevated when the level of zinc in the diet was increased 100 times (Reinstein et al. 1987). The change in the dietary level of zinc in the studies of Reinstein et al. (1987) and Finley and Johnson (1992) did not alter the activities of carboxypeptidases A and B. This suggests that pancreatobiliary secretion of zinc is likely not an important contributor to zinc homeostasis in the rat. However, the role of pancreatic secretions in zinc homeostasis may depend on the dietary intake of zinc and

the importance may vary between species. McClain et al. (1988) and Lee et al. (1990) concluded that pancreaticobiliary secretion of zinc is the major contributor to endogenous zinc and is important in maintaining zinc homeostasis in humans. Pancreatic secretion of zinc in pigs likely has a minor role in maintaining zinc homeostasis based on the relatively low level of daily zinc secretion in pancreatic juice compared to daily zinc intake.

In conclusion, the surgical method used to prepare pigs for collection of pancreatic juice has a large effect on the concentration of zinc in pancreatic juice and the activities of carboxypeptidase A and B. In addition, daily zinc secretion in pancreatic juice is relatively low compared to daily zinc intake in pigs fed diets which meet the zinc requirement. Zinc secretion in pancreatic juice parallels the secretion of protein and carboxypeptidase A and B. Zinc in pancreatic juice appears to be primarily associated with carboxypeptidase A and B.

E. Implications

The diurnal pattern of carboxypeptidase A and B peaked within 2 h feeding and therefore pancreatic secretion of these enzymes adapts to feeding regimen. Zinc secretion in pancreatic juice is relatively low compared to zinc intake and is therefore not likely an important contributor to zinc homeostasis in growing pigs fed diets which meet the zinc requirement. Zinc secretion in pancreatic juice may be more important when zinc intake is marginal or deficient.

Table 7-1. Daily volume of secretion and concentrations and flows of protein and zinc and specific and total carboxypeptidase A and B activities in pancreatic juice from pigs prepared with the Pouch or Catheter Method

Item	Units	Pouch Method	Catheter Method	SE[z]	*P* <
Volume	L $24h^{-1}$	2.63	4.09	0.47	0.05
Protein	g L^{-1}	7.15	3.25	0.20	0.0001
	g $24h^{-1}$	18.4	11.9	1.24	0.01
Zinc	mg L^{-1}	1.00	0.41	0.03	0.0001
	mg $24h^{-1}$	2.54	1.50	0.12	0.0001
Carboxypeptidase A	U L^{-1} x 10^{-3}	107.0	69.5	4.1	0.0001
	U $24h^{-1}$ x 10^{-3}	270.5	237.5	13.5	0.11
Carboxypeptidase B	U L^{-1} x 10^{-3}	571.8	208.2	30.2	0.0001
	U $24h^{-1}$ x 10^{-3}	1450.8	757.4	160.3	0.01

[z]Standard error of the mean, n = 108 except for 24-h means where n = 9.

Table 7-2. Correlation coefficients between protein concentration and secretion, zinc concentration and secretion and specific and total carboxypeptidase A and B activities in pancreatic juice from pigs prepared with the Pouch or Catheter Method[z]

	Pouch Method		Catheter Method
		Protein (mg L^{-1})	
Zinc (mg L^{-1})	0.68		0.62
Carboxypeptidase A (U L^{-1})	0.64		0.53
Carboxypeptidase B (U L^{-1})	0.43		0.62
		Zinc (mg L^{-1})	
Carboxypeptidase A (U L^{-1})	0.79		0.83
Carboxypeptidase B (U L^{-1})	0.45		0.60
		Protein (mg $2h^{-1}$)	
Zinc (mg $2h^{-1}$)	0.68		0.46
Carboxypeptidase A (U $2h^{-1}$)	0.64		0.42
Carboxypeptidase B (U $2h^{-1}$)	0.38		0.54
		Zinc (mg $2h^{-1}$)	
Carboxypeptidase A (U $2h^{-1}$)	0.73		0.54
Carboxypeptidase B (U $2h^{-1}$)	0.36		0.53

[z]n = 108 for the Pouch Method and the Catheter Method, respectively, all correlation coefficients are significant ($P < 0.01$).

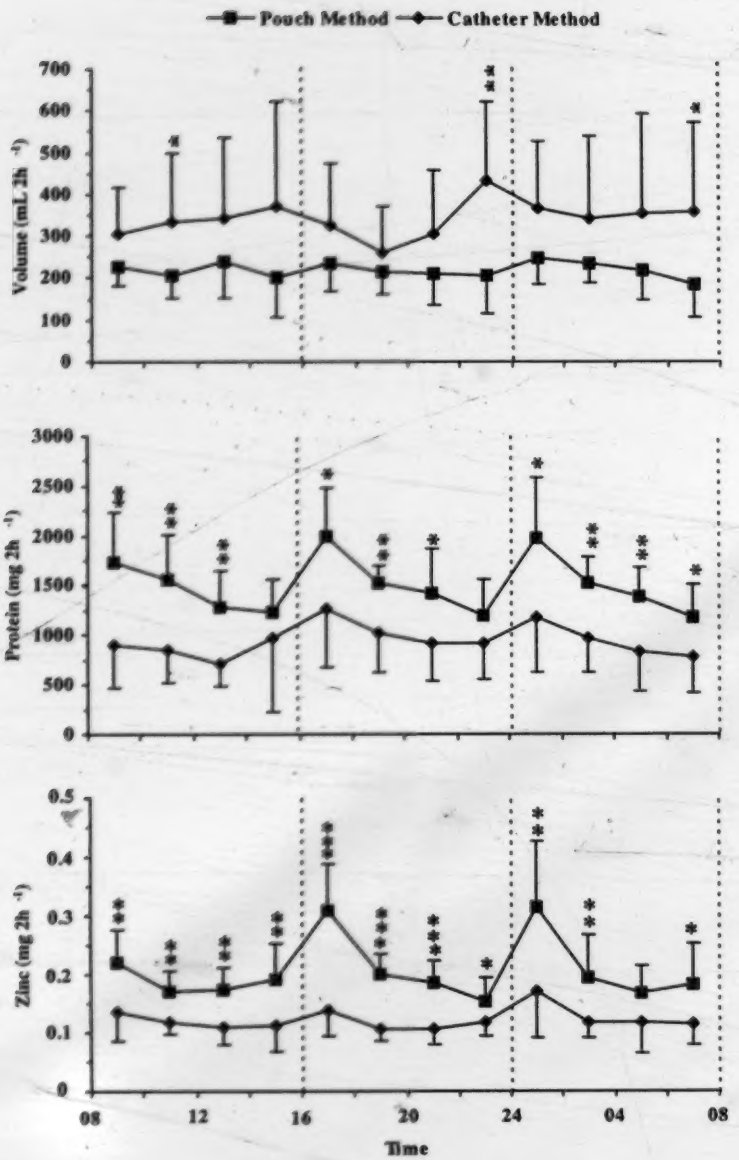

Fig 7-1. Diurnal pattern of volume and total secretion of protein and zinc in pancreatic juice from pigs prepared with the Pouch or Catheter Method. Secretion rates differ between methods: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Each point is the mean of nine observations and the standard deviation. The dashed vertical lines indicate feeding times.

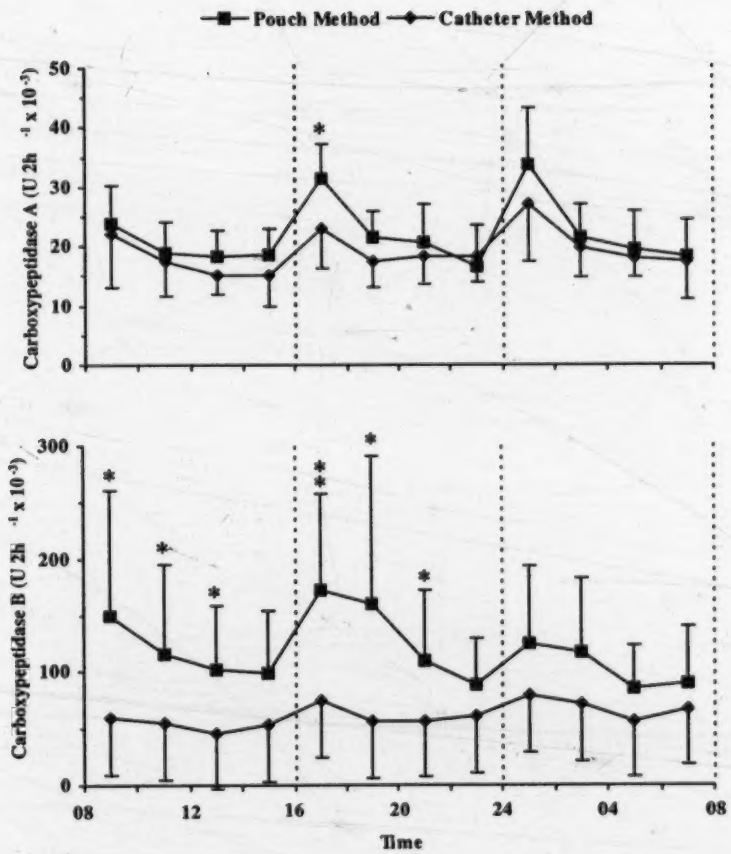

Fig 7-2. Diurnal pattern of total secretion of carboxypeptidase A and B in pancreatic juice from pigs prepared with the Pouch or Catheter Method. Secretion rates differ between methods: $*P < 0.05$, $**P < 0.01$. Each point is the mean of nine observations and the standard deviation. The dashed vertical lines indicate feeding times.

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CHAPTER 8

A COMPARATIVE STUDY OF TWO METHODS TO MEASURE AMYLASE, LIPASE, TRYPSIN AND CHYMOTRYPSIN ACTIVITIES AND THE EFFECT OF THAWING ON ENZYME ACTIVITIES IN PANCREATIC JUICE

A. Introduction

Reliable and reproducible methods for measuring enzyme activities in pancreatic juice are required for investigating the effect of changes in diet composition and the effects of different hormones, agonists and inhibitors on exocrine pancreatic secretions. One method is used to assay amylase, lipase, trypsin and chymotrypsin activities in porcine pancreatic juice in the Department of Agricultural, Food and Nutritional Science, University of Alberta (e.g., Hee et al. 1988; Chapter 2). A different method is used to measure these enzymes at the Danish Institute of Animal Science, Research Centre Foulum, Tjele, Denmark (Jensen 1994; Thaela et al. 1995). Therefore, it was decided to compare these methods using samples of pancreatic juice with low and high activities of amylase, lipase, trypsin and chymotrypsin. A summary of the methods used in the two laboratories is presented in Table 8-1. Very few studies comparing methods have been performed; two methods for determining amylase and lipase activities were compared by Goldberg and Wormsley (1970) and Schmidt et al. (1974).

Before analyses, refrigerated or frozen storage of samples is often required due to technical limitations, especially if a large number of samples are to be analyzed. Changes in enzyme activities during storage could affect the results and interpretation of an experiment (Legg and Spencer 1975; Muller and Ghale 1982; Kelly et al. 1991). Repeated freezing and thawing of samples may be necessary if the researcher decides to carry out additional analyses or another assay is performed at a later date. Very few studies have been conducted to examine the effect of storage conditions on enzyme activities in pancreatic juice. An additional concern is that thawing conditions are usually not mentioned in the literature and could affect enzyme activities.

The first objective of this study was to compare two different methods, referred to as Method A and B, for measuring amylase, lipase, trypsin and chymotrypsin activities in samples of porcine pancreatic juice with low and high enzyme activities. The second objective was to investigate the effect of a second freezing, storage and thawing on enzyme activities.

B. Materials and Methods

Source of Pancreatic Juice

Pancreatic juice used in this study originated from a previous experiment in which three growing pigs (average initial BW 32.4 kg) were surgically fitted with a catheter in the pancreatic duct (Chapter 5). The pigs were also fitted with a simple T-cannula in the duodenum to allow the return of pancreatic juice. A detailed description of the preparation of the pigs and sampling procedures was presented in Chapters 4 and 5. Three

proportionally pooled samples were prepared by pipetting 5% of the total volume collected each hour into a polyethylene bottle kept in ice until the completion of each 8-h sampling period. Ten minivials were labeled following each 8-h sampling period and 1 mL of the pooled sample was pipetted into each vial. Due to the number of samples and assays, it was necessary to freeze the samples prior to performing the first set of analyses.

Freezing and Thawing of Pancreatic juice

The freezing, storage, thawing and analytical procedures are summarized in Fig. 8-1. The vials and bottles were placed in a freezer at -80°C and stored for 2 wk. One set of subsamples (1 mL) was thawed at room temperature (approximately 22°C) and immediately placed into an ice bath prior to determining protein concentration and using Method A to assay amylase, lipase, trypsin and chymotrypsin activities. This was the first set of analyses of the 27 samples. Method A was used for the first set of analyses because it was the method being used in the laboratory at the Danish Institute of Animal Science to assay amylase, lipase, trypsin and chymotrypsin activities. Method B for assaying these enzymes was set up after the first set of analyses was carried out and was therefore only used for the second set of analyses. The effects of diet on volume, protein and bicarbonate secretion and specific and total activities of enzymes are presented and discussed in Chapter 4.

A second set of samples was prepared for the comparison of the two methods (A and B); the frozen pooled 8-h samples were thawed in warm water (30°C, for 10 to 15 min) and immediately transferred to an ice bath. Ten minivials were labeled and 1 mL of the pooled sample was pipetted into each vial. The vials and bottles were then placed in a freezer at -80°C and stored for 2 wk. One set of subsamples (1 mL) was thawed at room

temperature (approximately 22°C) and immediately placed in an ice bath prior to determining the concentration of protein and using Method A or B to measure enzyme activities. This was the second set of analyses of the 27 samples.

Chemical Analyses and Enzyme Activities

The concentration of protein in pancreatic juice, in both the first and second set of analyses, was measured according to procedures described in Chapter 4.

Enzyme activities were determined using the conditions summarized in Table 8-1 but some modifications were made to the original methods. In Method B for amylase, 100 mL of phosphate buffer was added to 1 g of soluble potato starch. This suspension was heated for 15 min in a water bath which was boiling. The tube containing the suspension was inverted every 5 min. In addition, distilled and deionized water (10 mL) was not added to the sample tubes following completion of the color reaction to avoid diluting the solution prior to determining absorbance.

In Method A for lipase, the assay was carried out in the presence of excess colipase. In Method B for lipase, preliminary analyses indicated that the addition of colipase did not result in a higher activity of lipase. Prior to measuring trypsin activity with either Method A or B, activation of trypsinogen to trypsin was carried out according to the method of Glazer and Steer (1977) with some modifications as described in Chapter 4. Trypsin activity was also determined in nonactivated pancreatic juice, with Method A in the first set of analyses. Activation of chymotrypsinogen to chymotrypsin, before using Method A or B, was performed according to procedures described by Glazer and Steer (1977) with some

modifications as outlined in Chapter 4. Chymotrypsin activity was also measured, using Method A, in nonactivated pancreatic juice in the first set of analyses.

Enzyme activity in pancreatic juice was expressed as specific activity: units (U) per liter or U per milligram protein. One U of enzyme activity was defined as the hydrolysis of 1 μmol of substrate in 1 min.

Statistical Analyses

One-way analysis of variance was used to determine the effect of the second freezing and thawing cycle of pancreatic juice on protein concentration and activities of amylase, lipase, trypsin and chymotrypsin using the GLM Procedure of the SAS Institute, Inc. (1988). Simple linear regression was used to examine the relationship between Methods A and B for measuring amylase, lipase, trypsin and chymotrypsin activities (SAS Institute, Inc. 1988).

C. Results

Amylase, lipase, trypsin and chymotrypsin activities determined with Methods A and B and the results of simple linear regression analyses are shown in Fig. 8-2. There was very good agreement between the activities of amylase and chymotrypsin measured with Methods A and B (R^2 = 0.95 and 0.90, respectively). There was more variability between the two methods with respect to trypsin activities (R^2 = 0.81). Similar results were obtained when the relationship between Methods A and B for amylase, chymotrypsin and trypsin activities were expressed in units per milligram protein (data not shown). There was a greater discrepancy between Methods A and B when lipase activities were determined (R^2 =

0.42, Fig. 8-2). Furthermore, when lipase activities were expressed per milligram protein, there were very large differences between the activities obtained with both methods (R^2 = 0.04, data not shown). This was due to additional variation introduced by the relatively weak relationship between lipase activity and protein concentration when Method A (R^2 = 0.55) or B (R^2 = 0.78) were used (data not shown).

In the first set of analyses of the samples, trypsin activity was not detected in nonactivated pancreatic juice and less than 1 U L^{-1} of chymotrypsin activity was detected (Chapter 5).

The effect of the second freezing and thawing cycle on the concentration of protein and enzyme activities, determined with Method A, in pancreatic juice is presented in Table 8-2. The second freezing and thawing cycle did not affect ($P > 0.1$) the concentration of protein or chymotrypsin activity even though these decreased by 12.3 and 29.0%, respectively. Amylase activity was also not affected ($P > 0.5$). However, the second freezing and thawing cycle decreased ($P < 0.01$) trypsin activity by 40.4% and lipase activity ($P <$ 0.001) by 82.9%.

The relationship between the second set of analyses and the first set of analyses for protein concentration, amylase, lipase, trypsin and chymotrypsin activities in pancreatic juice is shown in Fig. 8-3. The concentration of protein was not greatly affected by the second freezing and thawing cycle; however, greater reductions were observed when the concentration exceeded 6 g L^{-1}. This may have been a reflection, in part, of the substantial decrease in lipase activity, which did not exceed 500 U L^{-1} x 10^{-3} in the second assay, as well as in reductions in the activities of trypsin and chymotrypsin or other factors. Large

reductions in trypsin and chymotrypsin activities were observed once the activities exceeded 4500 and 4000 U L^{-1}, respectively. In contrast, amylase activity was very stable and did not decrease following the second freezing and thawing cycle. The relationships were similar when enzyme activities were expressed in units per milligram protein (data not shown).

D. Discussion

Very few comparative methodology studies have been carried out concerning the measurement of enzyme activities in pancreatic juice. The direct relationship between the two methods used in this study for measuring amylase, trypsin and chymotrypsin activities (Fig. 8-2) provides useful information to other researchers. Goldberg and Wormsley (1970) also reported that there was good agreement between two different methods, different from those used in this study, for measuring amylase activity in samples of duodenal aspirate.

The choice of a particular method will depend on the resources and equipment available. Outlying values from lipase analyses were omitted from the plot comparing the lipase activities obtained with both Methods A and B (Fig. 8-1). Part of the problem was likely due to incomplete removal of excess copper when the aqueous top layer was aspirated off the bottom layer of chloroform which contained copper salts of fatty acids released during hydrolysis of triacylglycerols by lipase (Schmidt et al. 1974). If traces of aqueous layer remain and are transferred to the second set of tubes (to which diethyldithiocarbamate will be added to produce the color reaction), the results will be greatly affected. This problem may be resolved if the chloroform layer is removed through the bottom of the tube following centrifugation to separate the layers. Another concern is the effectiveness of the

olive oil emulsion and it was also difficult to obtain a linear standard curve for stearic acid. This difficulty may have been due to traces of copper interfering with the color reaction as previously discussed. The photometric method of assaying lipase activity needs to be improved and investigated further. The results of this study do not agree with the comparative studies of Schmidt et al. (1974) in which there was a high correlation between lipase activity measured with the titrimetric and photometric method in duodenal aspirate, serum and pancreatin.

The reduction in the activities of trypsin, chymotrypsin and especially lipase (Table 8-2 and Fig. 8-3) is of practical significance showing that repeated freezing and thawing of pancreatic juice leads to an underestimation of enzyme activities. It would have been very useful to determine the impact of one freezing and thawing cycle on enzyme activities in fresh pancreatic juice, unfortunately this was not possible in this study because the enzyme assays could not be carried out immediately after collection. Very few studies have examined the effect of storage conditions on the activities of enzymes in pure pancreatic juice. Gorrill and Thomas (1967) reported that repeated freezing and thawing of nonactivated and undiluted pancreatic juice from calves, which had a catheter surgically inserted into the pancreatic duct, did not decrease trypsin and chymotrypsin activities; in some cases activities even increased. In the studies of Makkink et al. (1990), the Pouch Method was used, therefore, proteolytic enzymes were active and activation with either enterokinase or trypsin was not carried out. No differences ($P > 0.05$) in the activities of trypsin, chymotrypsin and lipase were observed when pancreatic juice was stored at either -20 or -80°C for 3 wk. However, when pancreatic juice was stored at 4°C for 3 wk, the

activities of trypsin, chymotrypsin and lipase declined ($P < 0.05$). The decline in enzyme activities was likely due to the presence of active pancreatic and bacterial proteolytic enzymes in the duodenal pouch (Makkink et al. 1990).

Many studies have investigated the effect of refrigerated or frozen storage on pancreatic enzyme activity in duodenal aspirate which likely contains active proteolytic enzymes. However, a description of the exact procedures of thawing duodenal aspirate prior to the assay have not been reported in detail. This is an important consideration that needs to be investigated further and if at all possible, thawing procedures should be standardized. Lipase activity did not decrease during storage at -15°C in the studies of Borgström and Hildebrand (1975). However, Kelly et al. (1991) reported that lipase activity declined steadily during storage at -20°C while chymotrypsin and to a lesser extent trypsin activities were quite stable for 7 wk. Amylase, lipase and trypsin activities in duodenal aspirate were quite stable at -20°C for 4 wk (Legg and Spencer 1975). However, when duodenal aspirate was stored at 4°C or room temperature, large decreases in lipase activity were observed but there were smaller decreases in amylase and trypsin activities (Legg and Spencer 1975).

Several modifications have been made to prevent a decline in enzyme activities in duodenal aspirate during storage but none of these approaches have been applied to the storage of pure pancreatic juice. A decline in lipase activity is prevented by adding albumin or casein, which act as alternative substrates for chymotrypsin and trypsin, or a chymotrypsin inhibitor (turkey egg white) to duodenal aspirate prior to freezing (Lake-Bakaar et al. 1980; Thiruvengadam and DiMagno 1988; Kelly et al. 1991). The addition of Trasylol (aprotinin, a trypsin inhibitor), glycerol or glucose reduces or prevents the

inactivation of amylase, chymotrypsin, lipase and trypsin during frozen storage of duodenal aspirate (Goldberg and Wormsley 1970; Gorrill and Friend 1970; Lake-Bakaar et al. 1980; Muller and Ghale 1982). However, whether or not Trasylol was removed from the samples prior to carrying out the enzyme assays was not reported. The use of Trasylol may interfere with the determination of trypsin activity and other proteolytic enzymes; further studies are required.

The decline in trypsin, chymotrypsin and lipase activities may have been due to bacterial contamination of pancreatic juice during collection and handling (Macfarlane et al. 1989). A small amount of proteolytic activity (< 1 U L^{-1} chymotrypsin and no trypsin activity) were measured in the first set of analyses.

Depending on the concentration of enzymes (reflected by the magnitude of enzyme activity present) the effect of storage may vary as was observed in this study (Fig. 8-3). Substantial inactivation or proteolysis took place prior to the second set of assays when the activity during the first assay exceeded 4500 and 4000 U L^{-1} for trypsin and chymotrypsin, respectively. Regardless of the results of the first lipase assay, the activity measured in the second assay was considerably lower. The small amount of chymotrypsin activity that was present may have been enough to degrade lipase. Lipase and other lipolytic enzymes are resistant to repeated freezing and thawing but are susceptible to hydrolysis by chymotrypsin (Kelly et al. 1991). Proteolysis or inactivation of lipase in pancreatic juice does not occur until the proteolytic enzymes have been active for approximately 1 h (Borgström et al. 1993). Furthermore, lipase is less resistant to inactivation than either trypsin or chymotrypsin (Pelot and Grossman 1962), which was confirmed in this study.

The stability of amylase activity in response to a second freezing and thawing cycle is clearly shown by the results presented in Table 8-2 and in the first and second set of assays shown in Fig. 8-3. The stability of amylase activity may be due to the fact that amylase contains divalent cations, such as calcium, which confer resistance to proteolysis (Stein and Fischer 1958). Protein concentration was not greatly affected by freezing and thawing despite the very large decreases in lipase, trypsin and chymotrypsin activities. This may indicate that these enzymes were inactivated by limited proteolysis.

In conclusion, the results of this study indicate that there is good agreement between two methods for measuring amylase, trypsin and chymotrypsin activities in pancreatic juice. However, until improvements are made in the photometric method for measuring lipase activity, use of the titrimetric method is recommended. Furthermore, based on the results of this study, repeated freezing and thawing of pancreatic juice is not recommended due to the sensitivity of lipase, trypsin and chymotrypsin to inactivation. However, amylase activity is not greatly affected by one freezing and thawing cycle, therefore it is valid to measure amylase activity in samples which have been frozen, stored and thawed more than once.

E. Implications

Reliable measurements can be obtained with either of the two methods used in this study for determining amylase, trypsin or chymotrypsin activities in pancreatic juice. However, lipase activity should be determined titrimetrically. A second freezing and thawing cycle should be avoided when the activities of lipase, trypsin and chymotrypsin are going to be measured.

Table 8-1. Substrates and conditions for determining the activities, with two methods, of enzymes in pancreatic juice from pigs prepared with the Catheter Method

Enzyme	Method	Substrate	Buffer	pH	°C	References
Amylase	A	Phadebas® amylase reagent[z]	154 mM NaCl, 0.2% BSA[y], 20 mM $CaCl_2$	7.0	37	Ceska et al. 1969
	B	Soluble potato starch[x]	10 mM NaCl, 20 mM Phosphate buffer	6.9	25	Rick and Stegbauer 1974; WBC 1988
Lipase	A	Tributyrin	1 mM Tris, 1 mM $CaCl_2$, 150 mM NaCl, 4 mM NaTDC[w]	7.0	22[v]	Erlanson-Albertson et al. 1987
	B	Stabilized olive oil emulsion	450 mM Triethanolamine HCl, 0.5 mM NaDC[u]	8.5	30	Schmidt et al. 1974
Trypsin	A	benzoyl DL-arginine p-nitroanilide	50 mM Tris, 50 mM $CaCl_2$	8.2	22[v]	Erlanger et al. 1961
	B	N_α-p-tosyl-L-arginine methyl ester	46 mM Tris, 11.5 mM $CaCl_2$	8.1	25	Rick 1974b
Chymotrypsin	A	N-succinyl-ala-ala-pro-phe p-nitroanilide	100 mM Tris, 10 mM $CaCl_2$	7.8	22[v]	DelMar et al. 1979; Lainé et al. 1993
	B	N-benzyol-L-tyrosine ethyl ester	80 mM Tris, 100 mM $CaCl_2$	7.8	25	Rick 1974a

[z]Pharmacia Diagnostics, Uppsala, Sweden. [y]Bovine serum albumin. [x]Code S 2630, Sigma Chemical, St. Louis, MO. [w]taurodeoxycholate. [v]Room temperature. [u]Deoxycholate.

Table 8-2. Effect of a second freezing and thawing cycle on the concentration of protein and the activities of enzymes, determined with Method A[z], in pancreatic juice from pigs prepared with the Catheter Method

Item	First Assay[y]	Second Assay[x]	SE[w]	*p* value	Change[v]	Percentage Change
Protein ($g\ L^{-1}$)	4.08	3.58	0.36	0.33	0.50	-12.3
Amylase ($U\ L^{-1} \times 10^{-3}$)	436	478	53.9	0.58	142	+9.6
Lipase ($U\ L^{-1} \times 10^{-3}$)	1290	220	140.1	0.001	1070	-82.9
Trypsin ($U\ L^{-1}$)	3682	2194	413.5	0.01	1488	-40.4
Chymotrypsin ($U\ L^{-1}$)	2346	1665	294.5	0.11	681	-29.0

[z]See Table 8-1.
[y]Pancreatic juice was frozen (-80°C), then thawed and analyzed.
[x]Pancreatic juice was frozen again (-80°C) then rethawed and analyzed.
[w]Standard error of the mean, n = 27.
[v]First assay - second assay.

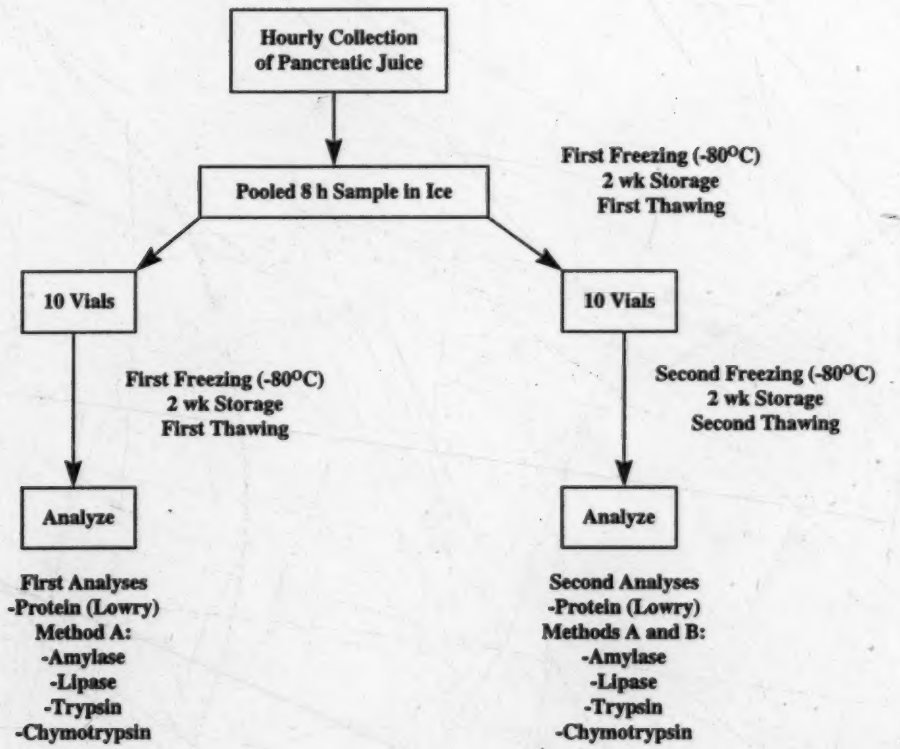

Fig. 8-1. Flowchart summarizing freezing, storage, thawing and analytical procedures.

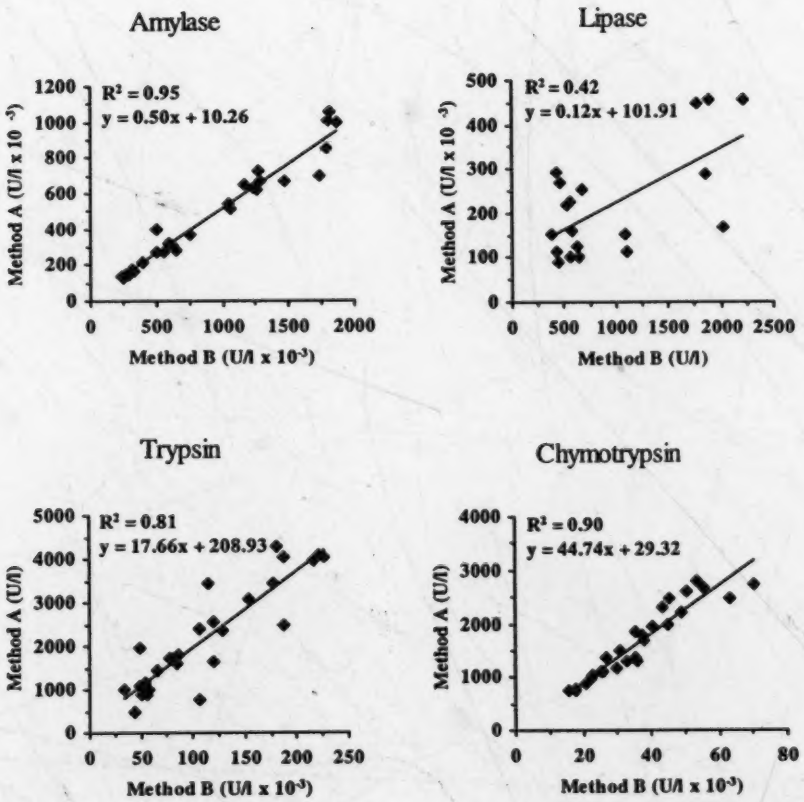

Fig. 8-2. Scatter plots (n = 27) and regression analyses between Methods A and B for amylase, lipase, trypsin and chymotrypsin activities in pancreatic juice from pigs, regression analyses and best fit line of the regression equation.

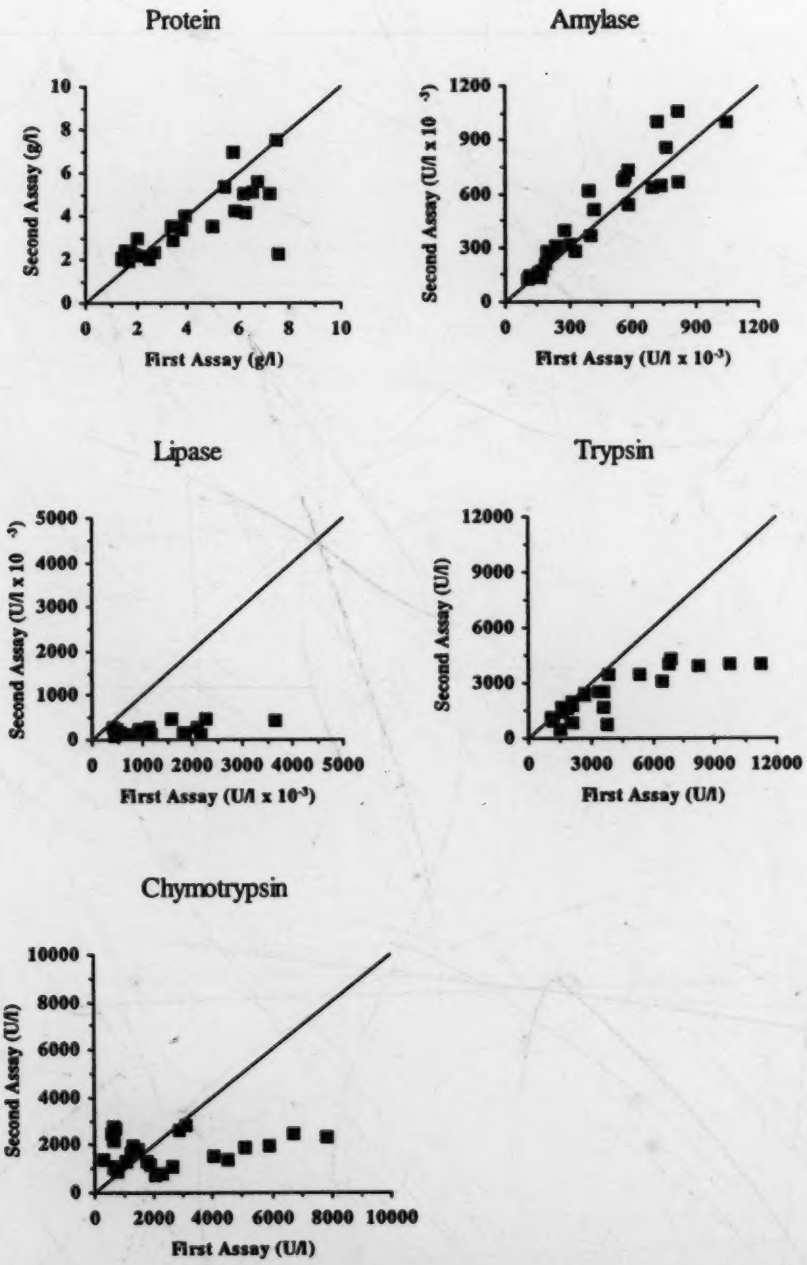

Fig. 8-3. Scatter plots of the second analyses, after freezing and thawing a second time, on the first set of analyses of protein, amylase, lipase, trypsin and chymotrypsin in pancreatic juice from growing pigs. Method A was used in both analyses. Unity lines are on the graphs for illustrative purposes to show the decline in enzyme activity or protein concentration during a second freezing, storage and thawing.

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

A. Discussion

The exocrine pancreas is a very important component of the digestive system and secretes enzymes that are required for digestion of protein, carbohydrate and fat. Exocrine pancreatic secretions adapt to changes in the levels of these nutrients in the diet (e.g., Corring 1980; Hee et al. 1988; Ozimek et al. 1995). A number of factors affect exocrine pancreatic secretion, including diet composition; interest in the effect of antinutritional factors and fatty acid composition on exocrine pancreatic secretion initiated the studies included in this thesis (Chapters 2 and 4). The flows of total, protein-bound and free amino acids in pancreatic juice may be affected by diet composition and may affect endogenous secretion of amino acids into the small intestine. Studies conducted in Chapter 3 investigated amino acid secretion from the pancreas. To study pancreatic function *in vivo*, surgical intervention is necessary to obtain pancreatic juice. Pancreatic secretions in response to various dietary factors may be affected by collection method. Therefore, studies were carried out to compare the two most commonly used methods, namely the Pouch (Zebrowska et al. 1983; Hee et al. 1985; Chapter 2) and Catheter Methods (Wass 1965; Pierzynowski et al. 1988; Thaela et al. 1995) to collect pancreatic juice from growing pigs (Chapters 5, 6 and 7). Pancreatic juice contains a relatively high

amount of zinc which is a component of the metalloenzymes carboxypeptidase A and B (Sullivan et al. 1974; Vallee and Falchuk 1993). However, there is a scarcity of information on the importance of pancreatic secretion of zinc to the maintenance of zinc homeostasis in the pig, therefore the secretions of zinc and carboxypeptidase A and B were investigated (Chapter 7). To assay enzyme activities in pancreatic juice, reliable methods are needed as activities may also be affected by storage and handling (Legg and Spencer 1975; Kelly et al. 1991). Therefore, in the final study (Chapter 8) two different methods for assaying enzymes were compared. In addition, the effect of one freezing and thawing cycle on enzyme activities was examined.

In the first study (Chapter 2) the effect of feeding diets containing whole fababeans and dry peas on exocrine pancreatic secretions of N, protein and enzymes in young pigs, prepared for collection of pancreatic juice with the Pouch Method, was investigated. Increasing production of fababeans and white-flowering spring peas has created interest among the feed industry to use these crops in diets for pigs. In the soybean meal and fababean diet (Experiment 1, 37.7% fababeans in the diet), a colored flowering spring variety of fababeans, cv Fibro, which contains a relatively high level of tannins (Mosenthin et al. 1993) supplied 50% of dietary CP and the remainder was supplied by soybean meal. Soybean meal was the sole protein source in the control diet. Peas were the sole protein source in diets used in Experiment 2. The pea cultivars selected were Ascona and Radley which have relatively low and high trypsin inhibitor activities, respectively (Leterme et al. 1992). Diet did not affect pancreatic secretions of N, protein or enzymes in Experiment 2. The lack of response to trypsin inhibitors in the

Radley pea diet was likely due to their relatively low level. Perhaps, there would have been an effect if the diets contained higher levels when a concentrated source of trypsin inhibitors was added to the diets. The pigs fed the soybean meal and fababean diet (Experiment 1) had higher ($P < 0.05$) specific trypsin activities in pancreatic juice than those fed the soybean meal diet. However, increased specific activity is of little nutritional significance as total enzyme activity reflects the quantity of enzymes that can participate in digestion; total trypsin activity was not affected ($P > 0.05$) by diet.

Further studies are warranted to examine the effect of tannins on pancreatic secretions in young pigs. Studies using diets containing a higher level of tannins are needed to investigate their mode of action. The levels in the current studies (Chapter 2) may have been too low to elicit a response. Tannins bind to proteins and enzymes making proteins resistant to digestion and inhibiting enzyme activity in the digestive tract (Marquardt et al. 1977; Liener 1980). However, a direct or indirect effect on pancreatic secretion has not been demonstrated.

The flow of total, protein-bound and free amino acids (AA) was determined in pancreatic juice from pigs in the first study (Chaptef 3). The percentage of protein-bound AA was 72.6% in Experiment 1 and 74.0% in Experiment 2. The most abundant protein-bound AA in pancreatic juice were aspartic acid, glycine, glutamic acid, serine, valine, alanine and leucine. The concentrations of these AA were related to the AA composition of the various enzymes in pancreatic juice. The most abundant free AA were leucine and lysine.

Further research is needed to quantify the origin of free AA in pancreatic juice and whether or not their presence has physiological significance to the small intestine. Free AA in pancreatic juice may arise from blood plasma, acinar cells, the epithelial cells of intralobular and interlobular ducts or epithelial cells of the main pancreatic duct. In addition, the release of AA from hydrolysis of pancreatic proteins by active trypsin and (or) chymotrypsin and action of bacterial proteolytic enzymes must be considered and investigated further. Other studies are needed on the reabsorption of protein-bound AA. There is a scarcity of information on the digestibility of pancreatic enzymes and their contribution to endogenous protein secretions. This is important in light of increasing interest in understanding which factors affect and contribute to endogenous protein (amino acid) secretions (Souffrant 1991; Bastianelli et al. 1996).

The effect of fatty acid composition on the volume of pancreatic juice secreted, protein and bicarbonate secretions and specific and total enzyme activities was studied in Chapter 4. Pigs were prepared with either the Pouch or Catheter Method and fed three diets containing 15% fish oil, rapeseed oil or coconut oil. Inclusion of polyunsaturated fatty acids in the diet has been shown to increase lipase activity in pancreatic homogenate (Deschodt-Lanckman et al. 1971; Ricketts and Brannon 1994). Simoes-Nunes (1986) reported that pigs fed diets containing 21% sunflower oil had higher lipase activity in pancreatic homogenate than pigs fed the same level of animal fat. However, in both groups of pigs, there was no effect of fatty acid composition on the specific or total activities of lipase.

Considering the limited information on the effect of fatty acid composition on pancreatic secretions in the pig, further research should be carried out. In addition, the relationship between enzyme activities in pancreatic homogenate and in pancreatic juice needs to be investigated further. The experiment of Simoes-Nunes (1986) should be repeated using pigs prepared for *in vivo* collection of pancreatic juice with the Pouch or Catheter Method.

The two commonly used methods, Pouch (Zebrowska et al. 1983; Hee et al. 1985; Chapter 2) and Catheter Method (Wass 1965; Pierzynowksi et al. 1988; Thaela et al. 1995), referred to as PM and CM, respectively, used for collection of pancreatic juice in Chapter 4, were evaluated in Chapters 5 an 6. An implicit assumption is that pancreatic secretion responds to changes in the amount and type of dietary nutrients regardless which surgical technique is used. However, the daily volume of pancreatic juice, and secretion of carboxyl ester hydrolase, colipase and trypsin were higher ($P < 0.05$) in pancreatic juice from CM than PM pigs. In contrast, amylase secretion was higher ($P < 0.05$) in PM pigs. The differences between the CM and PM pigs likely relate to the collection method. In CM pigs, bypassing the duodenal papilla, which may be controlled by secretin and cholecystokinin, or placement of a catheter in the pancreatic duct may increase secretion (Brannon 1990; Pierzynowski et al. 1995). In PM pigs, the construction of a duodenal pouch severs most of the extrinsic nerves leading from the stomach to the pancreas via the duodenum, therefore regulatory mechanisms may be affected and pancreatic secretions altered (Thomas 1959; Pierzynowski et al. 1988). Trypsinogen and chymotrypsinogen in pancreatic juice from PM pigs were fully active (Chapter 5). This

was verified by electrophoretic separation of proteolytic enzymes in samples of pancreatic juice from PM and CM pigs (Chapter 6). The presence of active proteolytic enzymes, responsible for inactivation or proteolysis, may have caused some of the differences in enzyme activities observed between the CM and PM pigs (Khayat and Christophe 1969; Borgström et al. 1993). On the other hand, amylase is relatively resistant to inactivation or proteolysis (Stein and Fischer 1958).

The two common methods used to prepare pigs for total collection of pancreatic juice have strengths and weaknesses and further evaluation of these methods is needed. The Catheter Method may be the more preferred method, it is less invasive and there is less interference with the regulatory mechanisms of pancreatic secretion and there are no confounding effects of active proteolytic enzymes. The Catheter Method is suitable for use in short term experiments similar to those in Chapters 4 and 5. The effect of the presence of a catheter in the pancreatic duct and bypassing the duodenal papilla on the release of gastrointestinal hormones and pancreatic secretions remains to be investigated. The Pouch Method can be used in long term experiments and morbidity and mortality may be lower than when the Catheter Method is used. Future research must address whether the Pouch Method affects regulatory and feedback mechanisms and secretions of cholecystokinin or secretin. Further investigations on whether or not special precautions or protease inhibitors need to be used during collection of pancreatic juice from animals prepared with the Pouch Method is required. However, the use of protease inhibitors may have implications for the determination of trypsin and chymotrypsin activities.

Pancreatic juice contains a higher concentration of zinc than other gastrointestinal secretions (Sullivan et al. 1974; Vallee and Falchuk 1993). Zinc is located in the active site and has a catalytic role in carboxypeptidase A and B which are the main sources of zinc in pancreatic juice (Pekas 1966; Berger and Schneeman 1986). The pattern of zinc secretion in pancreatic juice from growing pigs, prepared with the Pouch or Catheter Method, was parallel to the secretion of protein and carboxypeptidase A and B (Chapter 7). The percentage of daily zinc excreted in pancreatic juice was calculated to be 1.40 and 0.82% for PM and CM pigs, respectively. Therefore, the contribution of pancreatic zinc secretion to zinc homeostasis appears to be relatively low. However, it may be more important when zinc deficient diets are fed or when there is a marginal zinc deficiency which remains to be investigated. The contribution of pancreatic zinc secretion in pigs to total endogenous zinc excretion also remains to be determined.

To study pancreatic secretion of enzymes, reliable methods to assay enzyme activity are required. A direct relationship was observed between two different methods to determine amylase (R^2 = 0.95), trypsin (R^2 = 0.81) and chymotrypsin (R^2 = 0.90) activities. Therefore, either method can be used and selection will depend on resources available. The photometric method for assaying lipase activity needs to be improved and investigated further; there was a poor relationship (R^2 = 0.42) between the photometric and titrimetric method.

Prior to determining enzyme activities in pancreatic juice, samples often need to be kept refrigerated or frozen. Repeated freezing and thawing of samples may be necessary if the researcher decides to carry out additional analyses or if another assay is

performed at a later date. One freezing and thawing cycle of porcine pancreatic juice did not affect ($P > 0.1$) protein concentration, and chymotrypsin and amylase activities. However, trypsin and lipase activities decreased ($P < 0.01$) substantially. More research is needed on the effect of freezing, storage and thawing of pancreatic juice and on methods to prevent inactivation of enzymes during storage of pancreatic juice.

B. Conclusions

To summarize, the following conclusions can be drawn:

1. The inclusion of a colored-flowering variety of fababeans (37.7% of the diet), which had a relatively high content of condensed tannins, in a diet for young pigs does not affect exocrine pancreatic secretions of protein and total enzyme activities.

2. Exocrine pancreatic secretions of protein and enzymes in young pigs are not affected when a diet containing white-flowering spring peas (70.5% of the diet), with a relatively high content of trypsin inhibitors, is fed.

3. Pancreatic juice collected from young pigs contains free AA and depending on the AA being considered the free form makes a substantial contribution to total AA. In addition, the concentrations, flows and compositions of total, protein-bound and free AA in pancreatic juice are not affected by diet, sampling or experimental period. The AA composition of pancreatic juice and the daily flows of total, protein bound and free AA in

pancreatic juice are similar for younger (9.9 kg) and older (heavier, 22.6 kg) pigs and can therefore be used to estimate the composition and flow of AA in growing pigs.

4. Exocrine pancreatic secretions of lipase and colipase are not affected by the fatty acid composition when 15% fish, rapeseed or coconut oil are included in the diet. The total activity of carboxyl ester hydrolase is increased when pigs prepared with the Catheter Method are fed a diet containing 15% fish oil.

5. There are large and significant differences in the volume of pancreatic juice secreted, pH of pancreatic juice and specific and total activities of several enzymes when the Pouch and Catheter Methods are used. These differences may affect the response of the exocrine pancreas to changes in diet composition, feeding regimen or application of a particular hormone, agonist or inhibitor.

6. The Catheter Method may be a more suitable method, than the Pouch Method, to collect pancreatic juice in growing pigs. The Catheter Method is less invasive and most of the neural and hormonal mechanisms controlling exocrine pancreatic secretions likely remain intact. The confounding effects of active proteolytic enzymes are avoided when this method is used.

7. The Pouch Method may be suitable if secretions of amylase, lipase and chymotrypsin are investigated. These enzymes appear not to be greatly affected by the presence of active proteolytic enzymes in pancreatic juice.

8. The sensitivity of the Pouch and Catheter Methods, when these methods are used under the same conditions, to changes in diet, such as the adaptation to different levels of fat, protein and starch, remains to be determined.

9. The daily zinc excretion in pancreatic juice is relatively low compared to the daily zinc intake of pigs fed diets which meet the zinc requirement. Zinc secretion in pancreatic juice parallels the secretion of protein and carboxypeptidase A and B. Zinc in pancreatic juice appears to be primarily associated with carboxypeptidase A and B.

10. Either method for assaying amylase, trypsin and chymotrypsin activities can be used on samples of pancreatic juice (Chapter 8). However, until improvements are made in the photometric method, the titrimetric method should be used to assay lipase activity.

7. Repeated freezing and thawing of pancreatic juice is not recommended due to the sensitivity of lipase, trypsin and chymotrypsin to inactivation. Amylase activity is not greatly affected by one freezing and thawing cycle, therefore it is valid to measure amylase activity in samples which have been frozen, stored and thawed more than once.

C. Originality and Major Contributions to Knowledge

Some of the findings in this thesis represent an expansion of the frontier of knowledge in the related areas. The following points are claimed to be original contributions to knowledge.

1. The Pouch Method was adapted for use in young pigs; the cannula design and surgical procedures were modified and improved to facilitate total collection of pancreatic juice.

2. The inclusion of whole fababeans (37.7% of the diet), which had a relatively high content of condensed tannins, was shown not to affect ($P > 0.05$) the total secretion of amylase, lipase, trypsin and chymotrypsin in young pigs. Therefore, condensed tannins in whole fababeans do not likely have any detrimental effects on exocrine pancreatic secretion in young pigs.

3. When young pigs were fed diets containing dry peas (70.5%), with a relatively high content of trypsin inhibitors, exocrine pancreatic secretions of protein and enzymes were not affected ($P > 0.05$). Therefore, trypsin inhibitors in dry peas do not have any negative effects on exocrine pancreatic secretions in young pigs.

4. Free AA were shown to be present in pancreatic juice, depending on the AA being considered, free AA make a large contribution to total AA in pancreatic juice. The AA

composition and flow of total, protein-bound and free AA is similar for pigs differing in age (BW).

5. The modified design of the pancreatic pouch re-entrant cannula presented in Chapter 2 was applied for use in growing pigs (Chapter 4).

6. The catheter and simple T-cannula used to prepare young pigs for total collection and return of pancreatic juice (Pierzynowski et al. 1988; Thaela et al. 1995) were adapted for use in growing pigs (Chapter 4).

7. The inclusion of 15% fish, rapeseed or coconut oil, which differ in fatty acid composition, did not affect ($P > 0.05$) the secretion of lipase or colipase in pancreatic juice when either the Pouch or Catheter Method were used. However, the inclusion of fish oil increased ($P < 0.05$) carboxyl ester hydrolase activity when the Catheter Method was used.

8. The concentration of bicarbonate in pancreatic juice was determined using a new and simple method which can be readily used on fresh samples of pancreatic juice following collection.

9. Carboxyl ester hydrolase activity was determined in pancreatic juice from growing pigs.

10. Pigs prepared for collection of pancreatic juice with the Catheter Method (CM) secrete more ($P < 0.05$) pancreatic juice and the pH of pancreatic juice is higher ($P < 0.05$) than in pigs prepared with the Pouch Method (PM). Total amylase activity is higher ($P < 0.05$) in pancreatic juice from PM pigs. The total activity of carboxyl ester hydrolase, colipase and trypsin are higher ($P < 0.05$) in pancreatic juice from CM than from PM pigs. Trypsin and chymotrypsin in pancreatic juice from PM pigs are fully active, therefore, activation is not necessary.

11. The presence of active proteolytic enzymes in pancreatic juice collected from PM pigs is responsible, in part, for the lower ($P < 0.05$) carboxyl ester hydrolase, colipase and trypsin activities.

12. The presence of active proteolytic enzymes in pancreatic juice from pigs prepared with the Pouch Method was confirmed using electrophoresis.

13. The secretion of zinc and carboxypeptidase B in pancreatic juice is higher ($P < 0.05$) in pigs prepared with the Pouch Method than the Catheter Method.

14. Daily pancreatic secretion of zinc is relatively low compared to daily zinc intake in growing pigs fed a diet which meets the zinc requirement. The secretion of carboxypeptidase A and B peaked within 2 h of feeding and therefore adapts to feeding

regimen. Zinc secretion in pancreatic juice appears to be primarily associated with carboxypeptidase A and B.

15. A direct relationship was shown between two methods for assaying amylase, trypsin and chymotrypsin activities in pancreatic juice.

16. One freezing and thawing cycle causes large decreases ($P < 0.05$) in lipase, trypsin and chymotrypsin activities in pancreatic juice. On the other hand, amylase activity remained stable and was not affected ($P > 0.05$) by freezing and thawing.

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